A Study on Evaluation of Indigenous Microbial Consortium for Enhanced Decolorization of Textile Azo Dyes and Feasibility for Simultaneous Bioelectricity Generation in A Microbial Fuel Cell

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A Study on Evaluation of Indigenous Microbial Consortium for Enhanced Decolorization of Textile Azo Dyes and Feasibility for Simultaneous Bioelectricity Generation in A Microbial Fuel Cell

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We the below signed, after checking the dissertation mentioned above and the official record book (s) of the student, hereby state our approval of the dissertation submitted in partial fulfillment of the requirements of the degree of *Doctor of Philosophy* in *Chemical Engineering* at *National Institute of Technology Rourkela*. We are satisfied with the volume, quality, correctness, and originality of the work.

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This is to certify that the work presented in this dissertation entitled "*A study on evaluation of indigenous microbial consortium for enhanced decolorization of textile azo dyes and feasibility for simultaneous bioelectricity generation in a microbial fuel cell*", Roll Number 511CH110 is a record of original research carried out by her under my supervision and guidance in partial fulfilment of the requirements of the degree of Doctor of Philosophy in Chemical Engineering. Neither this dissertation nor any part of it has been submitted for any degree or diploma to any institute or university in India or abroad.

Prof. Susmita Mishra

I dedicate this thesis to

my parents

for their constant support

and

unconditional love

Declaration of Originality

I, Adya Das, Roll Number- 511CH110 hereby declare that this dissertation entitled " *A study on evaluation of indigenous microbial consortium for enhanced decolorization of textile azo dyes and feasibility for simultaneous bioelectricity generation in a microbial fuel cell* " presents my original work carried out as a doctoral student of NIT Rourkela and, to the best of my knowledge, contains no material previously published or written by another person, nor any material presented for the award of any other degree or diploma of NIT Rourkela or any other institution. Any contribution made to this research by others, with whom I have worked at NIT Rourkela or elsewhere, is explicitly acknowledged in the dissertation. Works of other authors cited in this dissertation have been duly acknowledged under the section "Reference". I have also submitted my original research records to the scrutiny committee for evaluation of my dissertation.

I am fully aware that in case of any non-compliance detected in future, the Senate of NIT Rourkela may withdraw the degree awarded to me on the basis of the present dissertation.

December 30, 2016 ***Adya Das***

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ABSTRACT

Extensive use of synthetic azo dyes in various colored industries and the proven toxic and aesthetic effect of these dyes and their degradation metabolites on environment makes the proper treatment of these colored effluent essential before discharging them into the outside waterbodies. Biological treatment methods are taking attention now-a-days as it is proven to be the cheap, eco-friendly and highly efficient treatment method for dye effluent in industrial scale as compared to the other available treatment methods. Organisms isolated from contaminated regions are believed to be more efficient in treatment of any xenobiotic and recalcitrant compounds as they are acclimatized to the toxic effect of the pollutants.

The present work emphasises on decolorization and degradation of different textile azo dyes (Reactive green-19 (RG-19), Remazol navy blue (RNB), Reactive Red-198 (RR-198) and a synthetic dye mixture (SDM) comprising of these three dyes using bacteria isolated from textile industry effluent. Three bacterial strains showing highest dye decolorization capacity were screened and identified as *Bacillus pumilus* HKG212 (GenBank Accession Number: KJ741252.1), *Zobellella taiwanensis* AT1-3 (GenBank Accession Number: FJ999669.1) and *Enterococcus durans* GM13 (GenBank Accession Number: KC213474.1) using 16s rRNA molecular analysis tool. *Bacillus pumilus* HKG212 was found to show highest decolorization capacity among the three isolates achieving 89% decolorization for RG-19, 95% decolorization for RNB and 94% decolorization for RR-198 dye within 24hr of incubation. However, all the three strains were found to be highly tolerant to higher dye concentrations achieving 80-95% decolorization for three model dyes in a wide range of dye concentration (i.e 50 mg/L to 1000 mg/L). Effect of different physico-chemical parameters on decolorization efficiency of the isolates revealed that, ambient temperature, neutral to slightly alkaline pH and static or anaerobic incubation condition favours the growth and decolorization potential of the organisms.

Isolated bacteria species in consortium showed high dye degradation potential as compared to pure strain. Consortium M12C comprised of *Bacillus pumilus* and *Zobellella taiwanensis* while consortium MA12C included *Bacillus pumilus*, *Zobellella taiwanensis* and *Enterococcus durans*. Dye decolorization potential of both the consortium has been evaluated for the decolorization of RG-19 dye. “MA12C” consortium was found to be more efficient decolouriser as compared to “M12C” consortium. Hence taking into consideration the real time application of this consortium, decolorization efficiency of “MA12C” has been evaluated for the decolorization of SDM. Synergistic effect of important process parameters was optimized using Response surface methodology and Box-Bheken design to maximize the decolorization potential of both consortiums. The optimum condition obtained for RG-19 decolorization using “M12C” were as follows: temperature, 32.04°C; pH, 8.3 and Yeast Extract concentration, 1.16 g/100 mL. Under this optimized condition, RG-19 bio-decolorization followed first-order kinetics and decolorization rate increased with increase in initial dye concentration. The double reciprocal plot for kinetics yield maximum

decolorization rate, V_{max} = 120.48 hr^{-1} and half saturation constant, K_m = 1674 mg/L for RG-19 removal using M12C.The optimal combination of variables obtained for SDM decolorization using MA12C was temperature 29.909 °C, pH 8.290 and yeast extract concentration 1.888 g/100mL. Dye decolorization kinetics of SDM states that, under optimized condition MA12C consortium was able to achieve maximum decolorization rate V_m of 68.96 h^{-1} when SDM concentration was greater than K_m i.e. 683.144 mg/L.

Anaerobic degradation of azo dyes results in formation of toxic amines which need to be treated in aerobic environment. However, slow anaerobic degradation kinetics makes the two-step approach of azo dye treatment laborious. In present study we have developed a laboratory scale integrated MFC-aerobic system to achieve enhanced decolorization and complete mineralization of RNB with concomitant production of biogenic electricity using MA12C consortium. A number of reports are available showing use of MFCs for efficient removal of azo dyes, however, most of these studies includes activated sludge or pure electrogenic microorganisms as inoculum. To the best of our knowledge, this could be the first report suggesting the use of a defined consortium developed from pure cultures isolated from dye contaminated wastewater as inoculum in MFCs for treatment of azo dye. Biodegradation of RNB dye has been confirmed through different analytical techniques such as HPLC, FTIR and GC-MS. Phytotoxic study confirms the non-toxic nature of the effluents released from each stage of the integrated treatment process.

Overall outcome of this study concludes that, microbes isolated from dye contaminated wastewater can be utilized for achieving enhanced azo dye degradation kinetics in anodic compartment of MFCs and simultaneous production of bioelectricity.

Keywords: *Azo dye; Biodegradation; Microbial Fuel Cell; Optimization; Kinetics; Consortium; Toxicity.*

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List of Abbreviations

MSM	Mineral salt medium
RSM	Response Surface Methodology
ANOVA	Analysis of Variance
HPLC	High Pressure Liquid Chromatography
GC-MS	Gas Chromatography Mass Spectroscopy
FTIR	Fourier Transform Infrared Spectroscopy
FESEM	Field Emission Scanning Electron Microscope
NCBI	National Center for Biotechnology Information
BLAST	Basic local alignment tool
PCR	Polymerase chain reaction
MEGA-5	Molecular evolutionary genetics analysis software v.5
EPA	Environmental protection agency
OD	Optical density
ppm	Parts per Million
rpm	Rotation per Minute
SDM	Synthetic dye mixture
SWM	Synthetic waste water medium
MFC	Microbial fuel cell
WHO	World health organization

Chapter 1

Introduction

In recent years the large scale industrialization and its associated impact on environment and biodiversity pose threat to the society. In India, textile industries contribute towards the largest economy of the nation. Since the discovery of first synthetic dye in 1856, advancement in synthetic chemistry together with rapid industrialization of textile production have done colossal damage to the aquatic environment in the form of toxic textile dye effluent. As more than 100,000 commercially available dyes [1] are used in various process of textile manufacture including dyeing and printing most of which are toxic, carcinogenic and recalcitrant in nature [2–4]. Textile industries consume maximum percentage of the synthetic dyes (around 56%) of world total annual production [5]. Textile industries uses large amount of water in every textile processing operations and waste water generated during these processes contains 5-10% of unfixed dyes [6]. It has been reported that, 10-50 mg/L of dye present in textile effluents which becomes problematic while considering large production units [7,8]. Continuous release of dye contaminated waste water without proper treatment contaminates soil, ground water, sediments and surface water etc. Colored effluents, when released into the nearby water bodies it affects the light and oxygen penetration decreasing the photosynthetic rate which ultimately affects the flora and fauna of aquatic eco-system. Above 1 mg/L concentrations, color becomes noticeable and textile waste water are reported to have more than 300 mg/L dye concentrations [9,10].

There are many structural varieties of dyes with respect to the type of chromophore, such as azo, anthraquinone, acridine, arylmethane, cyanine, phthalocyanine, nitro, nitroso, quinone-imine, thiazole or xanthene dyes. Azo dyes are the largest and the most diverse group of synthetic dyes accounts for 60-70% which are widely used in textile industries.

Permanence of these synthetic dyes in water is the main cause of pollution by textile effluent. The complex aromatic structure of these dyes makes it recalcitrant to degradation and conventional treatment methods are found to be ineffective for treatment of such colored effluents. Hence, removal of color is the main issue in treatment of these

colored wastewater. Several strategies being employed for the removal of color from the industrial effluent, before discharging them into the main water bodies which includes various physical, chemical and biological methods of treatment [11–14]. Efficiency of various physical and chemical methods of treatment such as adsorption, membrane filtration, chemical precipitation and flocculation, photolysis, ion pair extraction, oxidation with hydrogen peroxide (Fenton's reaction) etc. have been evaluated for the removal of color from industrial effluent. However, application of each of these methods found to have several shortcomings as they are costly, lack of effective color reduction and produce large amount of sludge which again produces an obvious disposal problem [15–17]. Moreover, because of their high stability against light, temperature and oxidizing agents, most of the dyes form textile effluents escape from such conventional treatment processes and persist in the environment for a long time. Bioremediation technology provides an effective, cheap and eco-friendly method to remove the color from dye containing wastewater [18,19].

Bioremediation is the method to remove or neutralize pollutant from a contaminated site with the use of biological systems such as microorganisms and plants. Microorganisms utilize various Carbon and Nitrogen sources for their growth and energy need. Various microorganisms use several organic pollutants as their energy source and microbial metabolism breaks down those pollutants into nontoxic intermediates. Sometimes organisms are unable to utilize the pollutant as their sole carbon source due to the complex nature of the pollutants. However, in some cases the metabolic pathway followed by microbes for their normal growth and development becomes useful for breakdown of some pollutants. Microorganisms have the potential to readily adapt to changing environment and this property of microbes makes them useful for the treatment of various toxic industrial waste. A wide variety of microorganisms have been reported for their potential to decolorize and degrade toxic dyes effluent [13,14,20]. A number of reports are available on oxidation of different textile dyes using various fungal species and their enzymes (e.g. laccases, peroxidases etc.) [1,21], however longer growth phase of fungus makes this approach time consuming. Short growth period and highly versatile nature of bacterial species makes them more useful in bioremediation of different pollutants. Although dye decolorization with pure bacterial cultures have been reported as

effective tool by various researchers, but these are not found to be highly effective for the treatment of actual industrial effluent and are highly dis-advantageous from cost and operational point of view in comparison to consortium of indigenous microorganisms [22]. Use of bacterial consortium may provide more realistic and efficient solution rather than pure culture. Hence trend must be shifted towards the use of bacterial consortium compared to pure cultures. Several mixed microbial cultures have been reported to be more efficient for color removal from dye effluent as compared to pure cultures [22–25]. However, continued search of new organisms and technologies are required to make these process of bioremediation more industrially feasible and ecofriendly.

Although there are wealth of information available in the literature about the use of different pure culture, co-cultures and mixed consortium of organisms capable of decolorizing various individual dyes, use of these findings for industrial application becomes difficult due to the complexity of mixtures of dyes present in textile wastewater. Therefore, potential of new isolated organisms for decolorization of mixture of dyes need to be evaluated and more study should be focused on the same.

The large chemical structure and highly charged nature of azo dyes hinders the penetration of these molecules into the cellular interior. Hence biological reductive degradation of azo dyes occurs due to the electron released during the microbial metabolism in the extracellular environment. Azo dyes are resistance to biodegradation under aerobic condition [26], however under anaerobic condition they undergo reductive degradation leading to formation of colorless aromatic amines [27]. Aromatic amines produced during these processes are toxic in nature and need further treatment and aerobic method of treatment has been found to be effective for the mineralization of these toxic intermediates [28,29]. Hence complete mineralization of textile azo dyes into non-toxic intermediates through bioremediation requires sequential anaerobic-aerobic treatment system [30–32]. However, the kinetics of anaerobic degradation of azo dye is found to be very slow which limits the use of this two-stage treatment approach. Therefore, a solution to enhance the rate of anaerobic degradation is essential to make this process industrially feasible.

Microbial fuel cell is an emerging technology suggested by many researchers for the treatment of different industrial wastewater with added advantage of harnessing electrical

energy [33]. Microbial fuel cell (MFC) is a device that convert the energy stored in chemical bonds of various organic compounds into electrical energy through the metabolic activity of microorganisms [34,35]. However, the reports available till date suggest that the, amount of electricity produced from MFC system is very low for any kind of practical applications. Hence, in current scenario researchers now motivated not only to use MFCs for the production of bioenergy, but also as an effective method for treatment of different industrial wastes.

Dual chambered MFCs are the commonly practiced MFC systems used in the laboratory which consists of a cathode and an anode chamber separated by a proton exchange system (generally a membrane). Organic matters get oxidized in the anodic chamber with the help of anodic microbial flora and the electrons produced during this process are transferred through an external circuit from anode to cathode. The protons migrate from the anodic chamber to the cathodic chamber through the proton exchange system and combine with oxygen and electron in the cathodic chamber to form water [36]. A number of microorganisms were reported to be used successfully in MFC system to catalyze the oxidation of organic substrates and simultaneous production of energy in the form of electricity [37].

Anaerobic environment of MFC anode can provide suitable environment for azo dye reduction. Successful decolorization of azo dyes and simultaneous production of electricity have been achieved in MFCs using different readily biodegradable carbon sources [38,39]. Unlike other anaerobic systems, MFCs utilize oxygen as the final electron acceptor which has a high redox potential. Hence it could be expected that, the metabolic rate of microorganisms must be higher in MFC system as compared to normal anaerobic digestion which can be beneficial to enhance the kinetics of azo dye reduction.

On summing up, increasing rate of industrialization and regulations enforced for release of industrial effluents, the demand for more efficient and less time consuming wastewater treatment systems is increasing. Biological method of treatment has been proved to be most effective and ecofriendly method of treatment for complete removal of toxic recalcitrant dye molecules from wastewater stream. Dye contaminated wastewater has been found to be an effective source of dye decolorizing microbial strains. Hence identification of more efficient organisms and study of different factors affecting their

efficiency of color removal is necessary for the development of a successful bioremediation system. Pure strains show different characteristics and efficiency of color removal when present in consortium. Therefore it becomes imperative to study the behavior of consortium while studying its dye decolorization efficiency under different environmental conditions. Optimization of process parameters is an important aspect while studying biodegradation of any pollutant. To achieve maximum efficiency of decolorization at industrially feasible environment it is necessary to have idea about the optimum conditions of process parameters affecting the efficiency of microorganisms. Kinetics of dye decolorization is another important aspect affecting the overall performance of bioremediation of dye contaminated wastewater. Exo-electrogenic nature of anodic microorganisms utilized in MFCs and the electrochemically active environment are found to be very effective for degradation of azo dye and speed up the kinetics of decolorization.

1.1 Motivation and Hypothesis

Effectiveness of a bioremediation process for the removal of any pollutant depends upon the survival, adaptability and activity of the organisms in the contaminated environment. Textile waste water has been reported to be a brilliant source of dye decolorizing microbial strains. The organisms isolated from dye contaminated sites are found to be highly tolerant to toxic effect of high dye concentrations, hence can be used effectively for the bioremediation of colored effluent released from various textile processing units [40–42]. Taking into consideration, the versatile nature of textile effluent, it has become necessary to continually search for new potential microbial strains and development of new strategies and technologies for effective removal of color from such wastewater. As discussed earlier, azo dyes constitutes 60-70% of total synthetic dye used in various dyeing industries, hence primary objective of this study is being motivated towards the removal of different azo dyes using biological treatment method. Though various pure microbial strains has been used by many researchers for effective removal of color from textile effluents, mixed and various defined microbial consortium were found to be more efficient to degrade the complex dye structures [22–24]. Taking the above facts into consideration, it can be expected that, individual and consortium of microbes comprising

of indigenous isolated acclimatized strains would be an effective option to achieve enhance color removal.

As the complex nature of azo dyes makes it difficult for microorganisms to use them as their sole carbon source, hence it becomes necessary to evaluate different co-substrates and microbial media components to support microbial metabolism which in turn will affect color removal efficiency of the organisms. Apart from culture media, various physico-chemical parameters are also found to affect microbial metabolism and hence the overall efficiency of the process gets affected. To achieve maximum efficiency of color removal in industrial level, it is essential to optimize the various process parameters and to study their synergistic effect on the process of biodecolorization.

Study of biodegradation kinetics is an important aspect need to be studied in any kind of bioremediation process. Rate of degradation of pollutant signifies the overall effectiveness of the process. Biodecolorization kinetics using single microbial strains has been reported by many researchers [43,44]. However, more studies should be done on decolorization kinetics using consortium of organisms.

Anaerobic degradation of azo dyes leads to formation of aromatic amines which need to be further treated due to their obvious toxic nature. Hence, sequential anaerobic-aerobic method of treatment of azo dyes is recommended by many researchers for complete mineralization of azo compounds into non-toxic intermediates. However, slow anaerobic degradation kinetics of azo bond slows down the overall process.

Use of microbial fuel cells (MFCs) for wastewater treatment is gaining interest of many researchers worldwide. Treatment of various xenobiotic compounds in MFC system has been reported in many literature including azo dyes [38,45,46]. However, most of these studies include use of anaerobic sludge as inoculum for azo dye treatment. When activated sludge used as inoculum in MFCs, microorganisms which are involved in dye degradation and current generation are present in the sludge in small populations and other organisms may interfere in the overall process by sharing the available nutrient. Hence it is essential that the optimal consortium be enriched for successful removal of dye and simultaneous recovery of energy in the form of electricity. As MFCs use oxygen as the terminal electron acceptor, it enhances the rate of microbial metabolism and more

number of reducing equivalents are available for the reduction of azo bond and dye decolorization kinetics could be expected to be faster as compared to traditional anaerobic method of treatment.

1.2 Organization of Thesis

The prime objective of the work presented in this thesis was to select potent dye decolorizing bacteria from dye contaminated wastewater to achieve enhanced biodecolorization of different azo dyes and to study their capacity for producing biogenic electricity in microbial fuel cell system.

The thesis has been organized into five chapters. **Chapter 1** gives a brief introduction about the use of synthetic dyes in textile industries, their toxic and aesthetic effect to the environment and the necessity for treatment of such dye pollutant. The motivation and hypothesis behind the project has also been discussed in this chapter.

Chapter 2 represents a short review of the relevant literature about the synthetic dyes, their chemistry, aesthetic effects, available treatment methods with their advantages and disadvantages.

Chapter 3 describes the details of materials and methods involved in the present study. The chapter has been divided into different sections. Each section started with a brief description of the concept behind the study to be conducted followed by the materials and methods used. This chapter elaborately describes the key approaches such as sampling, enrichment and acclimatization, isolation and screening of dye decolorizing microbial strains, optimization of process parameters and development of an integrated MFC-aerobic system for azo dye treatment. Different analytical techniques used to analyze the degradation products have been discussed in details.

The details of results of the present study is described in **chapter 4** and this chapter has been divided into three sections. The first section starts with details on the isolation and identification of dye decolorizing microorganisms from dye contaminated wastewater. Results of dye decolorization studies and effect of individual process parameters on decolorization efficiency of the pure isolates have been reported.

The next section emphasizes on the decolorization study using two novel microbial consortium. The dye decolorization efficiency of the consortia were maximized through

response surface methodology based Box-Behnken design method. Kinetics of dye decolorization using the consortia were investigated.

Last section of the chapter describes the results of decolorization studies and simultaneous bioelectricity production potential of a developed consortium in a MFC-aerobic sequential reactor system. Study of dye degradation metabolites was analyzed to evaluate the complete detoxification of the model azo dye.

Chapter 5 presents the major conclusions drawn based on the results and discussions of the previous chapter. It also furnishes recommendations for future work in this relevant field.

References used in this thesis are compiled at the end of this thesis.

Chapter 2

Literature Review

2.1 Summary

This chapter will primarily focus on the application of synthetic dyes, their structure and possible environmental impact of dye containing wastewater, most specifically azo dye. Various presently available treatment methods for these colored effluents will be explored with their possible advantages and disadvantages. More emphasis will be given to biological treatment methods and use of different microorganisms and their efficiency to treat dye containing effluents. Available technologies and current advancements in the field of biological treatment methods has been briefly explored.

2.2 Dyes and History of Dyes

Dyes are the colored aromatic organic compounds which show affinity towards the substance and provide color to which it is being applied. Use of colors for dying different substances has been practiced for thousands of years. Dyes have wide applications in textile, paper and printing, pharmaceutical, cosmetics and food industries [3,47]. The first written record for the use of dyes was dated at 2600 BC in ancient China. Before the discovery of synthetic dyes, natural dyes obtained from animal, vegetables or mineral origin has been used as coloring agents. The discovery of man-made synthetic dyes in late 19^{th} century replaced the large-scale market for natural dyes. The first manmade synthetic dye was prepared by William Henry Perkin in 1856.

It is necessary for the dyes to get completely or at least partially soluble in which it is being put to before being applied to color textile, leather, paper etc. The dye molecules get attached to the surface of the materials by forming either a covalent bond or by means of physical adsorption. The part of the compound that is responsible for the color of dye molecule is called the chromophore. It contains elements like carbon, nitrogen, oxygen and sulphur, alternating double and single bonds in part of the molecule and absorbs light in the visible region thus giving the dye molecule its color. It is important to mention here that, for the development of color, the chromophore should be a part of the conjugate

system within the dye molecule. Based on the type of chromophore, the dyes are classified as acridine, anthraquinone, methane, azo, nitro, carbonyl etc. [3,18]. In addition to chromophore, the structure of dye molecule also contains auxochromes which when bonded to the chromophore of dye molecule, helps in intensifying the color of the chromogen. Important group of auxochromes include carboxyl group, sulfonate, amine and hydroxyl groups etc. [3,18].

It has been reported that, the annual worldwide production of synthetic dyes is more than 7×10^5 tons and nearly 10,000 structurally different dyes are used in different industries [18,47,48]. Hence it becomes necessary to have a complete knowledge about the structure and properties of different dyes for the development of a successful remediation method for treatment of dye containing effluents.

2.3 Types of Synthetic Dyes and Their Uses in Color Industry

Dyes can be classified in several ways. Each class of dyes differ from the other based on their chemical structure, chemical properties and way of interaction with the substrate molecules.

Textile industries consume majority of the dye stuff produced worldwide. Hence we are mainly focusing on different dyes and their level of classification based on application in textile dyeing process.

2.3.1 Classification of Dyes Based on Structure of Chromophore

Based on the chemical structure of chromophore, dyes can be classified into 20-30 different groups. However, the most important group of dyes include azo dyes (-N=N- structure), anthraquinone dyes (derivatives of >C=O and >C=C), triarylmethane dyes, phthalocyanine dyes (derivatives of >C=N), nitro dyes (based on the $–NO_2$ functional group) etc. Among these, azo dyes represent the most important class of dye molecule and contributes 70% of total annual production of dye worldwide [18,47].

2.3.2 Classification Based on Nuclear Structure of Dyes

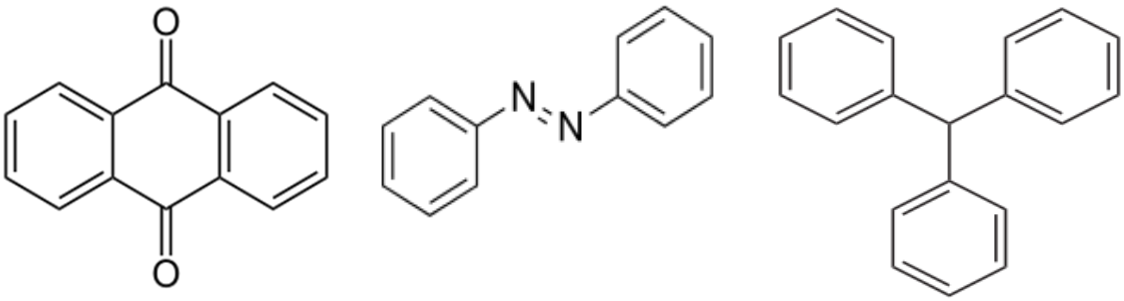
Based on nuclear structure dyes can be broadly classified as cationic (basic dyes) and anionic (acidic dyes) dyes.

- **Cationic dyes**

Cationic dyes are also known as basic dyes are characterized by $–NR_3^+$ or $=NR_2^+$ functional groups. Due to its positively charged nature, it reacts with negatively charged materials and mainly applied to acrylic fibers, silk and wool. These dyes are also found to be used in paper industries. However, cationic dyes are known to have poor shade stability and poor light fastness [49].

- **Anionic dyes**

Anionic dyes are water soluble acid dyes which is a salt of sulfuric, carboxylic or phenolic organic acid. They form ionic bond with the cationic site of fiber [50]. During dyeing process, acids are added to dyeing bath to maintain the protonated state of the fiber [51]. Acid dyes are mainly used to prepare synthetic food colors. Anionic dyes can be related to three basic structures namely anthraquinone type, azo type or triphenylmethane related (Figure 2.1).



(a)	**(b)**	**(c)**
Anthraquinone derivatives generally form blue dyes	Azobenzene derivatives generally form red dyes	Triphenylmethane derivatives generally form yellow or green dyes

Figure 2.1: Three basic structures of anionic dyes

2.3.3 Industrial classification of dyes

Industrially important dyes could be broadly divided into the following major classes based on their solubility, mode of interaction with the substrate and application. This include: Direct dyes, Reactive dyes, Acid dyes, Basic dyes, Solvent dyes and Disperse dyes.

- **Direct dyes**

Direct dyes attach to the fabric molecules directly by hydrogen bonding or by van-der-waals interaction. Majority of direct dyes are azo structure and belong to diazo or polyazo group of compounds. Direct dye has affinity towards cotton, silk jute, linen etc. As the bonding between the fiber and direct dyes are weak, they exhibit poor wash fastness, which leads to excessive loss of dye in the dyeing effluent [50].

- **Reactive dyes**

Reactive dyes attach to the fiber by forming a covalent bond with the appropriate textile functionality. Due to the strong covalent bond between the substrate and dye molecule, reactive dyes have good fastness and once attached, they are difficult to remove. Reactive dyes are mostly used to color cellulosic fibers, however they can also be applied for coloration of wool and nylon under weakly acidic condition. Mild dyeing condition and superior fastness favours the wide speared use of reactive dyes in textile industries.

- **Solvent dyes**

Solvent dyes are used by dissolving them in the target which can be a lipid or a non-polar solvent. Most of the solvent dyes belong to azo group of compounds, which after undergoing some molecular rearrangement have lost the ability to ionise. These dyes are used to color organic solvents, hydrocarbon fuels, waxes, lubricants, plastics and other hydrocarbon based nonpolar materials.

- **Disperse dyes**

Disperse dyes are the water insoluble dyes used for dyeing polyester and acetate fibers. They have small, planar and non-ionic structure with polar functional groups like $–NO_2$ and –CN. Disperse dyes undergo dying process at high temperature (about 130 $^{\circ}C$) which loosen the polymer structure, opening the gaps for dye molecules to enter that forms Van-

der-Waals interaction with fiber material. Small size of disperse dyes makes them highly volatile in nature, which causes poor color density. This drawback could be avoided by altering the structure of the dye molecule and making them more polar. Disperse azo dyes are one such group of compounds giving bright dyes.

2.4 Azo Dyes and Their Potential Threat to The Environment

As mentioned earlier in section 2.3.1, majority of industrially used synthetic dyes belong to azo group of compounds. Azo dyes are a large class of synthetic dyes, has a wide range of application in various industries such as textile, leather, paper, printing etc. due to its versatile color range better color fastness and stable chemical structure [52,53]. Textile industries are the largest consumers of these synthetic dyes and covers two-third of total dyestuff industry [53]. Hence, here we have tried to give more emphasis on the application of different azo dyes used in textile dyeing process and their aesthetic effects on environment.

2.4.1 Aesthetic Effects of Azo Dyes

Azo dyes are a group of synthetic dyes characterised by the presence of one or more chemical azo groups (-N=N-). In 1895, the workers involved in dye manufacturing were reported to have suffered from bladder cancer and since then number of studies have proved the toxic effect of azo dyes in human and animals [54]. The aryl rings attached to the azo groups generally belongs to benzene or naphthalene rings are the potential cause of toxic nature of azo dyes [17]. Presence of electron withdrawing group in azo dyes makes them recalcitrant towards various degradation processes [55]. However, reductive degradation of azo dyes under certain conditions produces a class of potentially dangerous chemical substances referred to as aromatic amines. Hence azo dyes and their degradation products are known for their toxic effect towards aquatic life, can be responsible for allergenic effects and are known to have carcinogenic and mutagenic effects on living organisms [4,56–58]. Expert authorities such as World Health Organisation (WHO) and other regulatory bodies worldwide restricted the use of certain azo dyes and strict guidelines have been implemented for detoxification of azo dye containing effluents released from various dyeing industries.

2.5 Current Available Technologies for Treatment of Azo Dye Containing Textile Waste Water

During textile processing, 10-50% of unfixed dyes enters to the effluent stream due to inefficient dyeing process [55]. Release of these colored effluents into the nearby waterbodies without proper treatment interferes in light penetration and obstructs oxygen penetration inside the aquatic eco system. Hence it becomes necessary to establish an efficient treatment method for the removal of toxic azo dyes from dye containing waste water and its complete detoxification, before being released to the environment. Available methods for removal of color from dye containing effluents can broadly be classified into three main categories such as: Physical methods, Chemical methods and Biological methods of treatment [48].

2.5.1 Physical and Chemical Methods

Important physical methods practised till date for the treatment of textile wastewater includes coagulation/flocculation, membrane filtration and adsorption [55,59,60].

Sulphur and disperse dyes are effectively removed through coagulation/flocculation method. However, this method of treatment is found to be ineffective for removal of acid, reactive, direct and vat dyes [59].

A number of reports proved membrane filtration technology to be an efficient tool for removal of dye from textile waste water effectively. Abid et. al (2012) reported the use of nanofiltration membrane for the removal of three different azo dyes acid red, reactive black and reactive blue and achieved 93.77%, 95.67% and 97% respectively [61]. Use of membrane filtration for treatment of textile effluents helps in separation of dye stuffs and simultaneous reduction of BOD and COD. However, high investment cost and generation of secondary waste stream limits the use of membrane for industrial scale application [59].

Adsorption is a well-practised method for removal for wide variety of dyes. Efficiency of adsorption depend upon the affinity between the adsorbent and dye molecule and the cost as well as the regeneration efficiency of the adsorbent [62]. A number of low cost adsorbents such as peat, fly ash, different agricultural wastes, natural clay etc. have been

investigated for their efficiency to remove dye from colored wastewater [63–66]. However, real time application of these adsorbents for the removal of dye in industrial scale is limited due to availability, regeneration capacity and the obvious disposal problem [59,67].

Widely used chemical treatment methods for azo dye removal includes chemical oxidation methods and the Fenton reaction method [59]. Chemical oxidation method makes use of different oxidising agents to degrade the chemical structure of dye molecules. Commonly used oxidising agents include ozone (O_3), hydrogen peroxide (H_2O_2), permanganate (MnO_4) etc. [59]. High reactivity of ozone with many azo dyes makes it an attractive method for azo dye treatment. However, short life span and high cost of ozone limits its practical application [67]. Advance oxidation process (AOP) makes use of single oxidant or combination of oxidants and found to be highly effective for color and COD removal compared to conventional chemical treatment methods [68]. The Fenton reaction method is effective in removal of both soluble and insoluble dyes and shows high COD removal efficiency [59]. Although AOPs are advantageous over conventional treatment methods and highly efficient for degradation of complex dye structure, the high cost of reagents and/or energy source and high sludge generation makes the process infeasible for real time application [59,68].

2.5.2 Biological Methods

Implementation of the available physical and chemical treatment methods for treatment of azo dyes in industrial scale suffers from several drawbacks being costly as they require more energy and chemicals, inefficient removal of azo dyes and their degradation products and produces large amount of sludge which again produces secondary disposal problem. Biological method of treatment is often an attractive alternative for treatment of azo dyes as it is inexpensive, eco-friendly and produces less sludge as compared to the available physical and chemical methods [59,69]. Microorganisms have the potential to acclimatize themselves to the toxic pollutant which leads to the development of new resistant strains with capability of degrading the toxic chemicals into less toxic intermediates. Decolourization of azo dyes through biological methods can occur due to biosorption, enzymatic degradation or a combination of both [70].

Biosorption is the process by which contaminants gets concentrate and bind onto the cellular structure of certain biomass. Biosorption of azo dyes by several microorganisms occurs due to the strong attractive force between the different functional groups such as amino, carboxyl, hydroxyl, phosphate and other charged groups present on their cell wall [71–73]. Various microorganisms such as fungi, bacteria, algae and yeast were reported to be involved in biosorption for removal of dyes [74].

Unlike biosorption, biodegradation and transformation of azo dyes by microorganisms occurs due to the action of various biotransformation enzymes which convert the toxic azo dyes into simpler non-toxic intermediates [69]. Presence of azo bond (-N=N-) and some electron withdrawing groups such as sulphonic groups (SO_3^-) in azo dyes makes them electron deficient and recalcitrant to biodegradation [75–78]. However, under certain conditions, microbial degradation of azo dyes occurs through reductases [59,79]. The successful implementation of a bioremediation system depends upon the adaptability and the response of the selected organisms towards the contaminant. A wide variety of microorganisms were reported to be capable of effectively decolorizing different dyes including fungi, bacteria, yeasts, algae etc. [80–85].

Fungi for Azo Dye Removal

Filamentous fungi are ubiquitous in nature. For their survival, fungi readily adapt their metabolism with change in carbon and nitrogen sources. The change in metabolic activity occurs through production of different intracellular and extracellular enzymes which helps in degrading complex organic pollutants [82,86,87]. Fungi shows the ability to degrade a range of organic compounds including different azo dyes by the action of their ligninolytic enzymes such as laccase, peroxidases etc. [88]. Most of the studies on degradation of azo dye using fungal culture involve different species of white rot fungi [82,87,88]. However, long growth phase and need for nitrogen limiting conditions limits the use of these organisms for treatment of azo dyes in industrial level [15,59].

Yeast for Azo Dye Removal

Very few reports are available on the use of yeast for the removal of azo dyes. Use of yeast for the treatment of azo dyes is more advantageous over the use of fungus because of its fast growth and the ability to survive in harsh environmental conditions [89]. A

number of reports are available on the use of yeast for successful removal of different dyes. Some ascomycetes yeast species follow enzymatic biodegradation mechanism for decolorization of different azo dyes [90], however, some yeast species were reported to show bioaccumulation and adsorption for removal of different reactive textile dyes [73,91].

Algae for Azo Dye Removal

Inherent resistance of algae towards toxicity of azo dyes makes them a favourable option for use in bioremediation of colored effluent [92–94]. Unlike bacteria and fungi, microalgae derive energy from sunlight and carbon and nitrogen from air which makes mass cultivation of algae inexpensive [95]. Similar to bacterial and fungal decolorization, microalgae also uses different enzymes to degrade the azo bond which results in formation of aromatic amines [95,96]. *Chlorella pyrenoidosa*, *Chlorella vulgaris* and *Oscillateria tenuis* were reported to degrade more than 30 different azo dyes into simpler amine intermediates [97]. In addition to enzymatic dye removal, algae were also reported to follow biosorption mechanism to remove different azo dyes [93,98].

Bacteria for Azo Dye Removal

Comparatively short growth phase, easy cultivation, ubiquitous and facultative nature of bacteria makes them the most frequently used microorganism for the treatment of azo dye containing textile effluent. Bacterial degradation of azo dyes occurs through reductive cleavage of azo bond (-N=N-) by azoreductase enzyme under anaerobic or facultative anaerobic conditions which results in formation of colorless toxic aromatic amines [99,100]. Sequential anaerobic-aerobic method of treatment has been suggested by many researchers for complete detoxification of azo dyes and its degradation metabolites [32,99,101,102]. Bacterial decolorization of azo dyes is gaining considerable interest in present time as bacterial species are capable of degrading a wide variety of azo dyes, is highly efficient in biodegradation and complete mineralization, requires less time and produce less sludge as compared to other biological methods [102–104].

Table 2.1: Comparison of azo dye decolorization efficiency of different microorganisms.

Microorganism	Decoloration process & Conditions	Initial dye concentration and Percent removal	Reference
Using Algae			
Spirogyra sp.	Adsorption;pH 2-10, 180 rpm, 20-50°C, 5hr	Direct brown (15mg/L), 70% color	[93]
Chlorella vulgaris, Lyngbya lagerlerimi, Nostoc lincki, Elkatothrix viridis and Volvox aureus	Degradation; pH 7, 25°C, 7days	(20 mg/L each) Methyl red (82% color), Orange II (47% color), G-Red (FN-3G) (59% color)	[105]
Oscillatoria curviceps	Degradation; pH 7, 100 rpm, 4°C, 8days	Acid black (100 mg/L), 84% color	[96]
Using Yeast			
Candida utilis	Adsorption; 150 rpm, 25°C, 10days	Remazol Turquoise Blue-G (50 mg/L), 82% color	[106]
Rhodotorula mucilaginosa	Adsorption; pH 3-6, 100 rpm, 30°C, 6days	Ramazol blue (389 mg/L), 96% color	[107]
Candida sp., Williopsis californica, Williopsis saturnus, Trichosporon porosum	Aerobic degradation, 250 rpm, 25°C, 24hr	Reactive yellow84, Reactive black 5, Reactive blue 221, Reactive red 141 (200 mg/L each), 96% color	[108]
Using Fungus			
Trametes versicolor	Degradation; pH 4.5, 800 rpm, 25°C, 3hr	Direct brown 2 (100 mg/L), 100% color	[109]
Phanerochaete chrysosporium	Degradation; pH 4.5, 150 rpm, 30°C, 72hr	Direct red 80 (20 mg/L), 100% color	[110]

Aspergillus niger	Adsorption and degradation; 100 rpm, 30°C, 24hr	(20 mg/L each) Acid red 151 (98% color), Orange II (84% color)	[111]
Trichoderma sp.	Adsorption; 24hr	Acid brilliant red B (100 mg/L), 100% color	[112]
Fusarium oxysporum	Degradation; 160 rpm, 24°C, 144hr	Yellow GAD (100 mg/L), 100 % color	[113]
Using Bacteria			
Sphignomonas paucimobilis	Aerobic degradation; pH 9, 30°C, 10hr	Methyl red (850 mg/L), 98% color, Degradation products not toxic	[114]
Micrococcus sp.	Aerobic degradation, pH 6, 35°C, 48hr	Orange MR (100 mg/L), 93.18% color	[115]
Bacillus sp., *Lysinibacillus sp.*	Degradation under static incubation, pH 7.2, 40°C, 12hr	Acid yellow 36 (200 mg/L), 100% color, Low phytotoxicity of degradation products	[116]
Shingobacterium sp.	Degradation under static incubation, pH 7, 30°C, 24hr	Direct red 5B (500 mg/L), 100% color	[117]
Alishewanella sp.	Degradation under static incubation, pH7, 37°C, 2% NaCl, 6hr	Reactive blue 59 (2500 mg/L), 95% color, Degradation products not toxic.	[118]

Decolourization of Azo Dyes Using Pure Bacterial Strain

Isolation of pure bacterial strains capable of degrading azo dyes was started since 1970s [119]. A variety of bacterial pure cultures such as *Proteus mirabilis*, *Pseudomonas luteola*, and *Pseudomonas sp.*, and isolated *Pseudomonas sp.* SUK1 has been reported to show promising results for decolorization of different azo dyes under anaerobic conditions [81,120,121]. Study of biodegradation using pure cultures ensures easy

reproducibility of the experimental data and hence interpretation of the mechanism becomes easier. Detailed knowledge regarding the mechanism of color removal using the pure bacterial cultures help in regulating the enzyme activity by producing modified strains. However, it has limited its application to R&D work and has little scope for industrial water treatment in large scale.

Use of Co-Cultures or Mixed Bacterial Consortium for Azo Dye Removal

Textile effluent composed of mixture of different azo dyes. Individual pure bacterial cultures are often found to be ineffective in complete mineralization of the complex dye structure and the toxic degradation products like aromatic amines. Maintenance of pure culture in industrial scale is another drawback which limits the use of pure culture for treatment of textile industry effluent. Since pure bacterial cultures are found to be less efficient in complete detoxification of real textile effluents, use of bacterial consortium may provide a comprehensive solution to this problem. A number of reports are available showing consortium of organisms to be more effective in color removal as compared to the pure strains [22–24]. Higher efficiency of consortium of organism as compared to the pure cultures may contributed to the fact that, in a consortium different organisms attack the dye molecule in different sites or the degradation products of one organism may get utilize by the co-existing strains which makes the overall process more efficient and faster [122,123]. The enzymatic activity of pure cultures gets affected by the presence of other strains which leads to different biocatalytic activity of consortiums as compared to the pure cultures. For the development of a consortium, the proportion of each organism plays an important role affecting the efficiency of the consortium for treatment of azo dyes.

Table 2.2: Comparison of azo dye decolorization efficiency of pure and consortium of bacteria

Bacteria Species	**Decolourization process**	**Initial concentration & Percent decolorization**	**Reference**
Pure Bacterial species			
Pseudomonas sp.	pH 7, 35°C, Static incubation, 70hr	Reactive blue 13 (200 mg/L); 83.2% color	[124]

Micrococcus glutamicus NCIM 2168	pH 6.8, 37°C, Static incubation, 42hr	Reactive green 19A (50 mg/L); 100% color	[125]
Escherichia coli JM 109(pGEX-AZR)	pH 9, 30°C, anaerobic, 12hr	Direct blue 71 (150 mg/L); 100% color	[126]
Brevibacillus laterosporus MTCC 2298	pH 7, 30°C, Static incubation, 48hr	Golden yellow HER (50 mg/L); 87% color	[127]
Pseudomonas aeruginosa	pH 7, 30°C, Static incubation, 24hr	Remazol orange (200 mg/L); 94% color	[128]
Bacillus fusiformis KMK5	pH 9, 37°C, anoxic, 48hr	Disperse blue 79 and Acid orange 10 (1.5 g/L each); 100% color	[129]
Bacterial consortium			
Pseudomonas aeruginosa, S. maltophila and P. mirabilis	Degradation; static incubation, pH 7, 45°C, 12hr	Direct black 22 (100 mg/L), 91% color	[130]
Sphingomonas paucimobilis, Bacillus sp., Staphylococcus epidermidis	Aerobic degradation; pH 7, 150 rpm, 37°C, 10hr	Congo red (750 mg/L), 100% color, degradation products not phytotoxic	[131]
Proteus vulgaris, Micrococcus glutamicus	Degradation; static incubation , pH 3, 37°C, 24hr	Reactive green 19 (50 mg/L), 100% color, Degradation products non-toxic	[132]
Alpha, beta and gamma proteobacteria	Aerobic degradation; pH 7, 27°C, 24hr	Reactive blue 59 (5000 mg/L), 100% color, products nontoxic	[133]

2.5.3 Mechanism of Biological Method for Azo Dye Removal

Azo dyes are electron deficient compounds characterised by (-N=N-) moiety. Biological decolorization of azo dyes occurs due to reductive degradation of the azo bond with the

help of azoreductase enzyme under anaerobic condition which results in formation of colorless aromatic amines [59]. The resulting amine intermediates are known to be recalcitrant to biodegradation under the same condition and need to be further treated under aerobic conditions [134]. Various aerobic and facultative anaerobic microbial consortia are reported to be able to decolorize different azo dyes under anaerobic condition [58,135]. Although most of them are capable of growing in aerobic condition, however, anoxic condition favours decolorization of azo dyes. Under anaerobic condition, azo dyes act as the electron acceptor and get reduced. Under aerobic environment, oxygen inhibits azo bond reduction by obstructing electron transfer from NADH to azo bonds [27,136].

Commercial azo dyes are generally have large molecular structure and are substituted with charged chemical groups. Large molecular size and highly charged nature of azo dyes hinder their penetration into non-polar biological membranes of microbes which becomes the principal rate-limiting factor for decolorization of azo dyes under anaerobic condition [59,137].

From the previous discussion, it is evident that, the available methods for treatment of azo dye possess certain drawbacks which limits their real time application. Hence there is a need to develop more efficient and eco-friendly alternative treatment for faster and complete degradation and detoxification of azo dye containing industrial effluents. Recently, Microbial fuel cells (MFCs) have been proposed as a promising alternative for achieving biodegradation of azo dyes and concomitant production of biogenic electrical energy [38,39]. Before discussing the recent advances and applications of MFCs for treatment of azo dye containing wastewater, a brief knowledge about the fundamentals of MFC technology is necessary.

2.5.4 Microbial Fuel Cells (MFCs)

Microbial fuel cell is an emerging technology which makes the use of microbial metabolism to degrade organic matter and concurrent production of energy in the form of electricity under anaerobic condition [37]. The mechanism of MFCs involves oxidation of organic substrates at the anode compartment by the action of electrochemically active

microorganisms. The electrons evolved during this process of microbial metabolism gets transfer to the cathode from anode via external circuit, thus producing electricity [138].

Real world application of MFCs for generation of electric power is limited because of their low power density level reported till date. However, MFCs are gaining attention of academic researchers as an effective tool for treatment of different industrial wastewater. Continual research is going on improving the performance of MFCs for treatment of various organic pollutants and simultaneous recovery of energy.

Mechanism of MFCs Performance

Under anaerobic condition, microorganisms used in MFCs convert the energy stored in organic substrates into usable electrical energy [37,139]. Bacteria gets its energy for survival in a two-step process. In the first step the organic matter gets oxidise, which then are transferred to either oxygen or nitrate and gets in the second step. When bacteria are grown in the absence of oxygen, the electrons generated by the bacterial metabolism can be transferred to a carbon electrode (i.e. anode). When these electrons move across the wire from anode to cathode, they generate current and voltage to make electricity [138]. Oxidation of substrates in anodic chamber of MFCs produces electrons and protons. Protons are transferred to the cathodic chamber through a proton exchange system (either a proton exchange membrane or a salt bridge) where it combine with the oxygen and electrons to produce water. Schematic diagram of a simple microbial fuel cell is shown in Figure 2.2.

Microbes Used in MFCs

Based on the mode of electron transfer from microbes to the electrode surface, MFCs can be broadly classified as mediator MFCs and mediator free MFCs. Most of the microbes are incapable of transferring electrons direct to the electrode surface and thus are said to be electrochemically inactive. In such cases electron transfer from microbes to the electrode occurs through mediators such as methyl blue, neutral red, thionine etc. [138]. However the mediators add additional cost to the MFC operation and are sometimes toxic to the anodic microflora. Some microbial species possess the ability to transfer electrons directly from cellular interior to the electrode surface; such microbes are referred to as electrochemically active and are mostly preferred to be used in MFCs. Such MFCs are

called mediator-free MFCs. Electrochemically active microbes were reported to be found in marine sediments, wastewater, soil and activated sludge [140,141]. A list of such microbes together with their substrates is shown in Table 2.3.

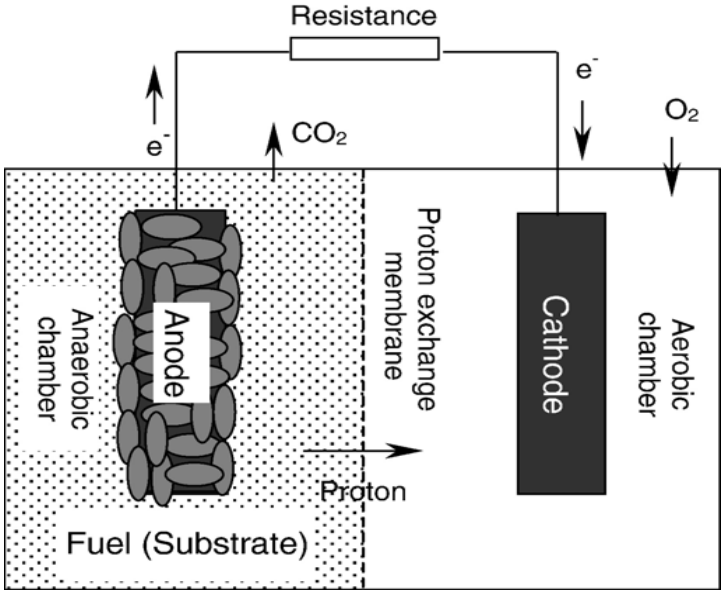


Figure 2.2: Schematic diagram of a simple double-chambered microbial fuel cell [37].

Table-2.3: Microorganisms used in MFCs

Microbes	Applications	Substrate	Reference
Aeromonas hydrophila	Mediator-less MFC	Acetate	[142]
Alcaligenes faecalis, *Enterococcus gallinarum*, *Pseudomonas aeruginosa*	Self-mediate consortia isolated from MFC with a maximal level of 4.31W/m2	Glucose	[143]
Geobacter metallireducens	Mediator-less MFC	Acetate	[144]
Rhodoferax ferrireducens	Mediator-less MFC	Glucose, Xylose, Socrose, Maltose	[36,145]
Shewanella putrefaciens	Mediator-less MFC	Lactate, pyruvate, acetate, glucose	[146,147]

Streptococcus lactis	Ferric chelate complex as mediators	Glucose	[148]

MFC Configuration and Operation

A typical MFC shown in Figure 2.2 consists of some basic components which is shown in Table-2.4. Based on the design and configuration, MFCs can be broadly classified into two categories i.e. dual-chambered MFCs and single-chambered MFCs. Dual chambered MFCs consists of an anaerobic anodic chamber and an aerobic cathodic chamber, separated by a proton exchange system. In the anodic chamber, substrate gets oxidised by microorganism (mostly anaerobic bacteria) generating electrons and protons. Electrons travel through the external circuit and proton migrates through proton exchange system to the cathodic chamber where they combine with oxygen to yield water.

Single chamber MFCs are simple in design and are cost effective in comparison to double chambered MFCs. They usually consist of an anodic chamber coupled with a cathode which is directly exposed to the air. The cathode is usually covered with porous plexi glass cover which allow oxygen penetration to the cathode surface. There is no severe necessity of proton exchange system for single chambered MFCs. Protons gets directly transferred to the porous air cathode from the analyte solution [149]. However, in some cases membranes are used in the form of membrane cathode assemblies to facilitate proton transfer to the cathode.

Table- 2.4: Basic components of MFCs [37].

MFC Elements	Constituents	Remarks
Anodic chamber	Glass, polycarbonate, plexiglass	Necessary
Cathodic chamber	Glass, polycarbonate, plexiglass	Optional
Anode	Graphite, graphite felt, carbon paper, carbon cloth, Pt, Pt black, reticulated vitreous carbon (RVC)	Necessary
Cathode	Graphite, graphite felt, carbon paper, carbon cloth, Pt, Pt black, RVC	Necessary

Electrode catalyst	Pt, Pt black, MnO_2, Fe^{3+}, polyaniline, electron mediator immobilized on anode	Optional
Proton exchange system	Proton exchange membrane: Nafion, Ultrex, polyethylene poly (styrene-co-divinylbenzene); salt bridge, porcelain septum, or solely electrolyte	Necessary

MFCs in Wastewater Treatment

The idea of using MFCs for treatment of industrial wastewater started in 1991 [150]. Industrial wastewater has been found to be a rich source of variety of organic compounds that can be utilised as fuel in MFCs [151–153]. It can be expected that, the power generated through the treatment of wastewater in MFCs can reduce the power requirement of the waste treatment plant to certain extent. In addition to that, the MFCs produce 50-90% less sludge as compared to the conventional treatment processes [154]. Use of MFCs for treatment of different wastes such as food processing wastewater, sanitary wastes, swine wastewater, corn stover etc. has been reported by various researchers [155–158]. Rabaey et al. (2006) used MFCs for to remove sulphide from wastewater [159]. In few recent studies, the application of MFCs for the treatment of different xenobiotic compounds such as p-nitro-phenol, petroleum hydrocarbons and different azo dyes was reported [45,46]. Upto 90% of COD removal [160,161] and coulombic efficiency as high as 80% has been reported in some cases [162].

2.6 MFCs for Azo Dye Treatment

Decolourization of azo dyes through microbial anaerobic reductive degradation is usually very slow. MFCs use oxygen as the final electron acceptor at the cathode which increase the metabolic rate of anodic microflora. In addition to that, operating conditions of MFC is also expected to have influence on the electron transfer rate from microorganisms to the azo dyes. Hence, kinetics of azo dye decolorization could likely to be faster in the electrochemically active environment of MFC [38,137]. Very few reports are available in the literature regarding the application of MFC technology for treatment of azo dyes. Li et al. (2010) studied the degradation of an azo dye Congo Red (CR) in an anaerobic-aerobic sequential reactor coupled with microbial fuel cell system [163]. However, they failed to

address the kinetics of CR degradation and influence of CR concentration on rate of decolorization. Mu et al. in 2009 demonstrated abiotic reductive degradation of Acid Orange 7(AO7) in MFC cathode. However, the rate of AO7 decolorization was very slow in the absence of external power supply [164]. Waheed et al. (2015) used a dual chambered MFC for studying the decolorization of a tetra-azo dye Direct Red 80 (DR 80) using anaerobic sludge as inoculum [39]. At the initial dye concentration of 200 mg/L, they achieved 85.8% color removal in 48 hr of batch MFC operation. However, they failed to report the kinetics of Direct Red 80 decolorization. Eustace et al. (2012) reported the decolorization kinetics of azo dye Acid Organge 7 (AO7) in a MFC anode by *Shewanella oneidensis* strain 14063. They were able to achieve more than 98% color removal within 30 hr of batch operation at all the tested dye concentrations ranging from 35 mg/L to 350 mg/L. Table 2.5 demonstrates the application of MFC technology for biological decolorization of different azo dyes along with the source of inoculum used [138].

Table-2.5: Different azo dyes and source of inoculum used in MFCs

Azo dye	**Source of Inoculum**	**Initial dye Concentration (mg/L)**	**% Color removal achieved**	**References**
Active brilliant red X-3B	Mixture of aerobic and anaerobic sludge	300	100% in 48h	[45]
Acid orange 7	Microbial consortium	0.06-0.24	78% in 1.44h	[164]
Methyl Orange	*Klebsiella pneumoniae* strain L17 from subterranean forest sediment	0.016	100% in 3h	[165]
Congo red	Anaerobic sludge from anaerobic digester of SiBao wastewater treatment plant	100	_	[163]

Model textile dyes	*Proteus hauseri* ZMd44	450–560	_	[166,167]
Amaranth	Microbial consortium	75	82.59% in 1h	[168]
Methyl orange	Anaerobic sludge Gaobeidian wastewater treatment plant	10–20	73.4% in 24h	[169]
Congo red	Mixture of aerobic sludge and anaerobic sludge collected from the Liede municipal wastewater treatment plant	300	90% in 170h	[170]
Congo red	Mixture of aerobic sludge and anaerobic sludge	300	98% in 36h	[171]
Acid Orange 7	Shewanella oneidensis strain 14063	35-350	98% in 30h	[38]

2.6.1 Effect of Operational Parameters on Dye Decolorization Efficiency of MFCs

- **Effect of Dye Concentration**

MFC performance gets affected by the initial dye concentration. Eustace et al. (2012) investigated the effect of Acid Orange 7 concentration on decolorization rate and MFC performance of a dual chambered MFC [38]. Their study showed that, with increasing AO7 concentration, decolorization rate decreases. The electrochemical performance of the MFC was found to be unaffected with low to moderate dye concentrations. However, higher AO7 concentration leads to notable reduction in power densities. This may be due to the increasing toxic effect of the dye and its degradation metabolites on anodic microorganisms with rising dye concentration. Similar finding was also reported by Waheed et al. (2015) for decolorization of Direct Red 80 in a dual chambered microbial fuel cell [39]. At 25 mg/L of Direct Red 80 concentration, 91.1% color removal was achieved which significantly decreased to 54.7% when the dye concentration has been

increased to 800 mg/L. However, they noticed a slightly decrease in current production with increase in dye concentration. These observations conclude that dye concentration is an important parameter need to be taken into consideration while evaluating the performance of MFC systems for treatment of azo dyes.

- **Effect of Dye Structure**

Chemical structure of dyes has significant effect on the performance of dye decolorization. Monoazo dyes are decolorized easily as compared to the polyazo dyes [76]. The position of electron withdrawing groups near to the azo bonds have significant effect on reductive cleavage of azo dyes which follow the order as para > ortho > meta. As ortho and para position withdraws electron from azo bond more efficiently through resonance, azo bond becomes more electrophilic to get reduced. However, higher steric hindrance at ortho position leads to slower decolorization as compared to para position [138].

- **Effect of External Resistance**

MFC external resistance influences the anode potential and hence the exo-electrogenic activity of anodic microflora gets affected. Large molecular weight of azo dyes obstruct the penetration of these dyes into cellular interior of anodic microorganisms. Therefore, the transfer of reducing equivalents to the azo dyes occurs extracellularly. Menicucci et al. (2005) reported that, the external resistance greatly influences the current and power generation in MFCs [172]. Eustace et al. (2013) studied the effect of external resistance on azo dye decolorization kinetics in MFCs [137]. Their study suggests that, an optimum external resistance affects the external electron transfer efficiency of the anodic microbes which in turn influence azo dye degradation kinetics. Under higher external resistance, it could be expected that more reducing equivalents will be available to the azo dye moieties, hence reduction of azo bond becomes faster. However, under much higher external resistances, fermentative and methanogenic microbes become dominant in MFCs and the environment becomes unfavourable for exo-electrogenic bacteria [173]. These studies suggest that, external resistance is an important parameter affecting azo dye reductive decolorization kinetics and power generation in MFCs.

- **Effect of Hydraulic Retention Time (HRT)**

Hydraulic retention time is an important parameter in any wastewater treatment processes as it affects the effluent substrate concentration. Li et al. (2010) investigated the effect of HRT on color removal in a dual chambered MFC [163]. They reported that, with increasing the HRT from 7.5 to 45 hr, color removal in anodic chamber increased from 35% to 82%. Maximum power density reached with HRT of 14.8 h. With increasing the HRT from 14.8 to 44.4 hr, substrate concentration in anodic chamber decreases, which increased the open circuit potential of anode from -431 to -238.8 mV. With decreasing HRT to 7.4 hr substrate concentration in cathode chamber increases, which caused a decrease in power density.

- **Effect of Co-Substrate**

Literature suggests that, different bacterial population requires different co-substrates for dye decolorization process. Similarly, in MFCs, the maximum achievable power density from a particular microbial community also depends upon the type of substrate utilised. Cao et al. (2010) studied the effect of different co-substrates on power density during decolorization of Congo red in a single chambered MFC [171]. They achieved highest power density of 103 mW/m^2 for glucose as co-substrate which is much higher as compared to that obtained for sodium acetate (85.9 mW/m^2) and ethanol (63.2 mW/m^2). Similarly, Sun et al. (2009) investigated the effect of different co-substrates for decolorization of ABRX3 and simultaneous production of electricity in a single chambered MFC [45]. They obtained maximum decolorization of ABRX3 with glucose followed by sucrose and acetate. Hence, selection of co-substrate is an important aspect for maximizing the efficiency of MFC systems.

- **Effect of MFC Elements**

Nature of cathode material greatly affect the maximum power production capacity of MFCs. Li et al. (2010) reported that, with the use of graphite-granular cathode, the power density of an MFC increased from 256.33 to 364.50 mW/m^2, however use of a carbon felt as cathode decreased the power density from 59.16 to 45.13 mW/m^2 [163]. Sun et al. (2012) investigated the effect of anode surface area on decolorization of Congo red and concomitant production of bioelectricity in a single chambered air cathode MFC [45].

MFC power output was found to be unaffected by the increased anode surface area, however dye decolorization got accelerated. Larger anodic surface area may provide more space for bacterial attachment which results in accelerated decolorization of Congo red dye. Therefore, choice of anode material and configuration of anode is an important parameter to achieve optimal performance of MFC systems.

- **Dye Decolorization Mechanism in MFC**

Anaerobic degradation of dye in MFC anode follows the same mechanism as anaerobic batch reactors yielding similar degradation products such as amines and sulfanilic acids. The color removal primarily occurs due to the reductive cleavage of azo bond rather than biosorption. Mu et al. (2009) studied the decolorization mechanism of AO7 in a bioelectrochemical system [164]. In the anodic chamber, electron and proton are produced from bacterial oxidation of substrate and are transferred to the cathodic chamber via the external circuit and the proton exchange membrane respectively. In the anodic chamber, dye decolorization occurs biologically through co-metabolism of anodic microbes. At the cathode, the azo bond breaks abiotically resulting in the formation of toxic intermediates.

2.7 Toxicity of Dye Contaminated Wastewater and Treated Effluent

The metabolites produced from anaerobic degradation of azo dyes are sometimes reported to be more toxic as compared to their parent dye. Different azo dyes and the amines produced from reductive degradation of such dyes were shown to have mutagenic and carcinogenic effects and the toxicity of these dyes depends upon their structure and position of the substituent molecules [69]. For example, 3-methoxy-4-aminoazobenzene shows strong hepatocarcinogenic effect in rats and mutagenic to bacteria, whereas 2-methoxy-4-aminoazobenzene is a non-carcinogen and a very weak mutagen in bacteria [174]. The biodegradation products of an azo dye Acid Voilet 7 by *Pseudomonas putida* in static condition leads to formation of 4’-aminoacetanilide and 5-acetamido-2-amino-1 hydroxy-3, 6-naphtalene disulphonic acid. These metabolites along with the parent dye were reported to have ability to induce chromosome aberrations and the inhibition of acetylcholinesterase [175]. Hence it becomes necessary for any kind of bioremediation strategies to evaluate the toxicity of the dye pollutant and its degradation metabolites

before implementing them for treatment of industrial effluent. Different methods have been implemented by various researchers to study the toxicity of various azo dyes and their degradation metabolites which includes phytotoxicity, mutagenicity, acute toxicity, genotoxicity etc. Phytotoxicity study have become widespread by most of the researchers as it is easy and inexpensive as compared to other methods. Some commonly used plant species for study of phytotoxicity includes *Phaseolus mungo* [25,176,177], *Sorghum vulgare* [176–178], *Triticum aestivum* [114,179] and *Oryza sativa* [180,181]. Acute toxicity has been evaluated using *Artemia salina* and *Artemia nauplii* [114]. *Allium cepa* root cells has been used to study cytotoxic and genotoxic nature of azo dyes and the degradation metabolites [178,182,183].

2.8 Sequential Anaerobic-Aerobic Method for Complete Detoxification of Azo Dye Containing Wastewater

As discussed earlier, anaerobic biological treatment of azo dyes has been proven to be the most feasible alternative among all the available treatment methods. However, reductive degradation of azo dyes under anaerobic condition produces toxic aromatic amines which are recalcitrant to biodegradation under the same condition and needs to be treated further aerobically [134,184]. Thus many researchers have suggested sequential anaerobic-aerobic systems for complete mineralization of azo dyes [32,101,185].

Treatment of azo dyes using MFCs follows the similar mechanism as the anaerobic degradation method yielding colorless aromatic amines as the degradation metabolites. Untreated aromatic amines may contribute to severe environmental toxicity and increased level of COD in the effluent. Very few studies have focused on integrated MFC-aerobic systems for complete degradation and detoxification of azo dyes and their degradation products [163,186]. However, in most of these studies mixed microbial population or activated sludge has been used as inoculum in MFCs and for the subsequent aerobic process. Mixed microbial population often comprises of variety of microorganisms, most of which are not involved either in dye degradation or generation of electricity process. On the other hand, sometimes they affect the overall efficiency of the process by sharing the available nutrient. Hence it becomes necessary for enrichment of the optimal electrochemically active microbial population having capacity to degrade azo dyes and simultaneously producing bio-electricity for achieving maximum efficiency.

2.9 Concluding Remark

Excessive use of synthetic dyes and the release of dye contaminated wastewater into the environment without proper treatment creates severe aesthetic and medical problems. Azo dyes contribute to the highest percentage of industrially used synthetic dyes which are reported to have toxic, carcinogenic and mutagenic properties. As regulations are becoming more stringent about treatment and detoxification of industrial effluent, there is an urgent need for continuous development of newer treatment methods which are technically feasible and cost-effective. Use of microbial degradation of azo dyes is gaining much attention, as these are cost-effective and eco-friendly as compared to various other practised treatment methods. Use of bacteria for decolorization of colored effluent has been reported to be advantageous among other microorganisms because of their fast growth rate and lower hydraulic retention time.

Anaerobic reductive cleavage of azo dye leads to formation of colorless toxic aromatic amines. Hence it is necessary to isolate organisms having capacity not only to degrade the azo dyes but also can degrade the toxic amine intermediates. Sequential anaerobic-aerobic method of treatment has been suggested as appropriate for complete detoxification of these azo dye contaminated colored wastewater. Since various physico-chemical parameters influence the efficiency of biological treatment methods, optimization of these parameters is essential.

Kinetics of biodegradation process is an important aspect in any kind of bioremediation process. Kinetics of anaerobic decolorization of azo dyes is slower which limits its application for treatment of industrial effluent. Application of MFCs for azo dye treatment has been reported by many researchers. Electrochemically active environment of MFCs is expected to be suitable for enhancing the decolorization kinetics of azo dyes. Decolourization of different azo dyes and simultaneous electricity generation has been tested with different pure bacterial strains which include *Enterobacter cancerogenus*, *Proteus hauseri*, *Acinetobacter johnsonii* etc. [165–167] and with enriched mixed microbial culture [170,171]. However, defined microbial consortium comprising of dominant microbial strains from dye contaminated sites could be expected to have higher dye removal efficiency as compared to the pure strains. Application of such microbial

consortium in MFCs could increase the efficiency of overall process. As most of the municipal WWTPs rely on the microbial component of the activated sludge process (which includes both aerobic and anaerobic treatment process), application of potential bacterial consortium in existing facilities can be targeted for bioremediation for efficient treatment of dye containing wastewater.

2.10 Objectives of Present Work

The aim of this study to evaluate the efficiency of indigenous microbes and their consortium for decolorization of textile azo dyes and test their feasibility for enhanced decolorization and simultaneous production of biogenic electricity using microbial fuel cell, with the following specific objectives:

- Isolation of bacterial strain from dye contaminated wastewater.
- Screening of potent organism with maximum dye degradation capability.
- Optimization of growth condition of the screened organisms and their characterization.
- Development of consortium from these screened organisms.
- Comparative study on decolorization efficiency of individual and consortium of bacterial species on individual azo dye solution as well as synthetic dye mixture.
- Optimization of process parameters to maximize the decolorization efficiency using statistical tool.
- Study of dye degradation kinetics using the developed consortia.
- Enhancement of degradation efficiency by developed consortium using microbial fuel cell.

Chapter 3

Materials and Methods

3.1 Isolation and Screening of Potent Dye Decolorizing Bacterial Strains and Study of Their Dye Decolourization Capacity

Various physicochemical methods like adsorption, membrane filtration, coagulation–flocculation, and oxidation are found to have limited applicability for treatment at industrial level because of high cost, lack of versatility and excessive sludge deposition which leads to secondary pollution problem [15,27,80]. Biological treatment method for textile dye effluent is technically attractive, eco-friendly and provides a cost effective alternative to other methods. Over the past decade, a wide variety of microorganisms have been reported for their potential to decolorize and degrade these toxic dye effluent [13,14,20], however there are few reports available on their application at industrial level. The effectiveness of these biological treatment methods using various microbial communities depends upon the survival and adaptability of microorganisms during the treatment processes. Textile wastewater sample are expected to have effective microbial consortia which are tolerant to higher dye concentrations and are likely to have capacity to degrade different textile dyes [40,187]. Considering these facts, textile wastewater sample was chosen as the source of microorganisms for this study.

The present investigation was undertaken to isolate potent dye decolorizing bacterial strains from dye contaminated wastewater and to study their maximum dye decolorization potential. This study also emphasises the effect of different process parameters on decolorization potential of screened pure bacterial strains for a better outcome.

3.1.1 Sample Collection

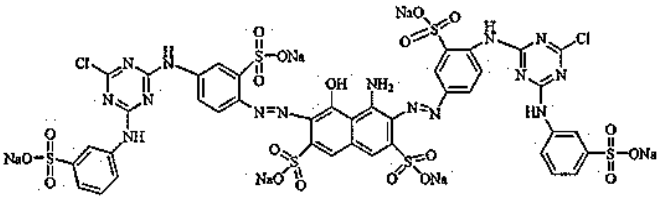
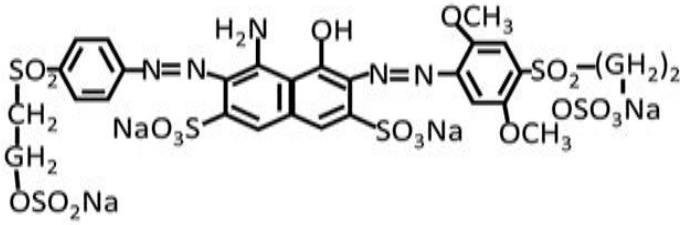
Textile wastewater samples were collected from industrial outfalls of textile industries in and around Viwandi, Mumbai. Waste water samples were collected in sterile glass-screw cap bottles from different locations and sampling sites were selected on the basis of the

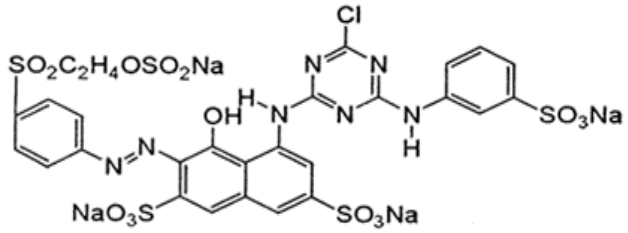
distribution of outlet from textile units. Collected water samples were brought to the laboratory in sterile condition and was stored at 4^0C to inhibit further microbial activity until further use.

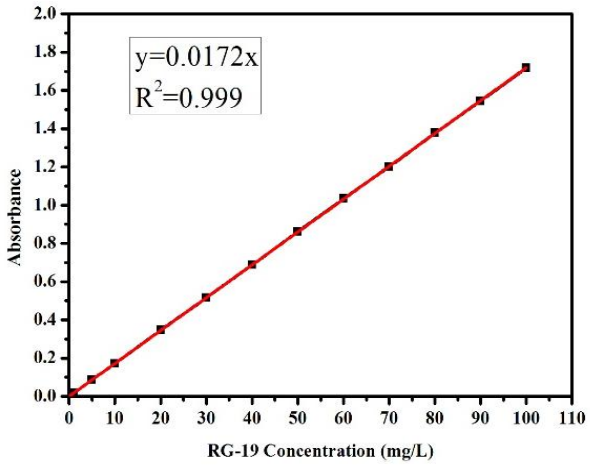
3.1.2 Target Textile Dyes

Three different textile azo dyes namely Reactive green-19 (RG-19), Remazol navy blue (RNB) and Reactive red-198 (RR-198) were selected as the model dyes for this study. The dyes were selected based on their structure and recurrent usage in textile industries. Dyes were purchased from a textile dye whole seller from Kolkatta, India and are of commercial grade with 98% ≥ purity. Structure and chemical properties of all the three dyes are given in Table-3.1. The stock solutions of all the three dye (concentration 10,000 mg/L) were prepared with distilled water and desired concentrations of working solutions were prepared by further subsequent dilutions. Equal volumes of RG-19, RNB and RR-198 was used to prepare the dye mixture solution used in simulated waste water. The calibration curves were prepared (Figure 3.1) for each dye and was found linear up to 100mg/l of concentrations with R^2 ≅1.000.

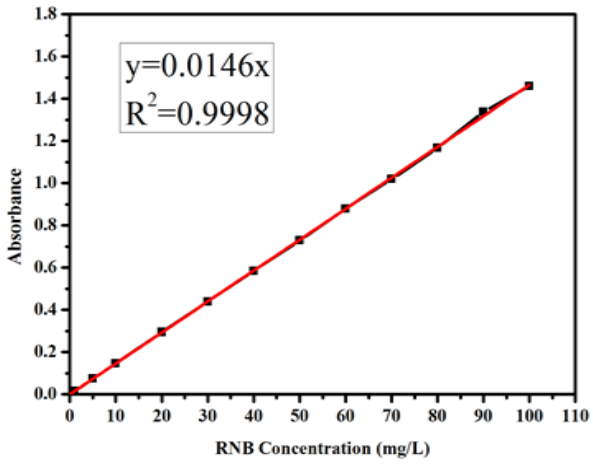
Table 3.1: Structure, Molecular weight & Absorption maxima of model dyes

Dye	Molecular structure	Molecular weight	Absorption maxima (nm)
Reactive green-19		1418.93	635
Remazol navy blue		847.3	597

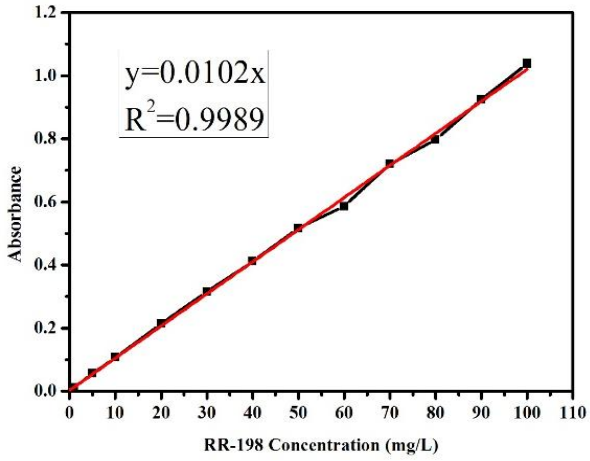
Reactive red-198		984.21	521



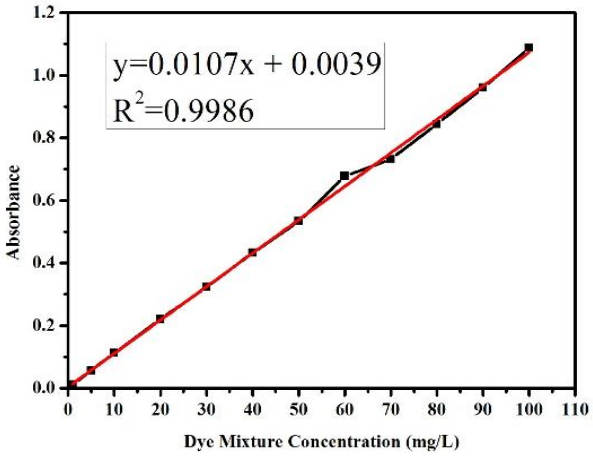
(a)



(b)



(c)



(d)

Figure 3.1: Calibration plots of model dyes (a) for Reactive green-19 (RG-19), (b) for Remazol Navy Blue (RNB), (c) for Reactive Red-198 (RR-198), (d) for dye mixture

3.1.3 Bacterial Culture Media

Initial isolation, screening of potent dye degrading bacteria and batch decolorization studies for effect of various process parameters on decolorization capacity of isolated strains was carried out using nutrient medium [188,189].The isolated organisms were stored in nutrient agar slants. Nutrient broth ingredients per liter peptone 10 g, sodium chloride 10 g, yeast extract 5 g, pH 7.6. Nutrient agar ingredients per liter peptone 10 g, sodium chloride 10 g, yeast extract 5 g, agar powder 30 g, and pH 7.6 [189]. The culture media and the glassware were autoclaved at 15 psi (121 $^{\circ}$C) for 20 min prior to the experiments and aseptic conditions were maintained wherever necessary.

3.1.4 Chemicals Used in Experiments and Analysis

Ultrapure water was obtained from a Millipore Milli-Q® system. Milli-Q® water was used for preparation of all analytical standards, microbial culture media and reagent preparation for all analytical techniques. All the chemicals and organic solvents used were of analytical grade and were obtained from Merck. Ingredients of bacterial culture media was obtained from Himedia.

3.1.5 Enrichment, Acclimatization and Isolation of Potent Dye Degrading Microorganisms

Textile waste water sample was initially exposed to enrichment, followed by acclimatization with increasing concentrations of dye mixture solution in nutrient broth medium. 250 ml Erlenmeyer flasks each containing 100 ml of dye containing medium was inoculated with 10 ml of wastewater sample and incubated at 30 $^{\circ}$C for 48hr under static condition. Repeated transfer of 10 ml aliquots were carried out until complete decolorization of the broth to isolate stable dye decolorizing strains. The acclimatised culture so obtained were serially diluted with autoclaved saline Millipore water (0.85% NaCl) from 10^{-1} to 10^{-7}. From each dilution, 100 µl of culture was spread on different designated nutrient agar plates containing dye mixture using sterile glass spreader and incubated at 30 $^{\circ}$C. After 48 hr of incubation, a number of colonies showing zones of decolorization were isolated and further purified by repeated streaking on nutrient agar plates.

3.1.6 Screening and Identification of Isolated Organisms

Morphologically distinct colonies showing zone of decolorization on dye containing nutrient agar plates were isolated and further screened based on their highest decolorization ability by performing dye decolorization assay with all the three model dyes in liquid medium (i.e. RG-19, RNB & RR-198) using UV-VIS spectrophotometer (UV-VIS spectrophotometer-3200, LabIndia Instruments Pvt Ltd, India). Organisms showing highest dye decolorization potential were selected and preliminary morphological identification was carried out through FE-SEM analysis.

Selected organisms were sent to Xcelris labs Ltd, Ahmedabad, India for 16s rDNA identification. The genomic DNA was isolated from the isolated pure cultures and quality was evaluated on 1.2% Agarose Gel. The fragment of 16S rDNA gene was amplified by PCR using 8F and 1492R from isolated DNA. A single discrete PCR amplicon band of 1500 bp was observed (Figure 3.2). The PCR amplicon was purified and further processed for the sequencing. Forward and Reverse DNA sequencing reaction of PCR amplicon was carried out with **8F** and **1492R** primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer.

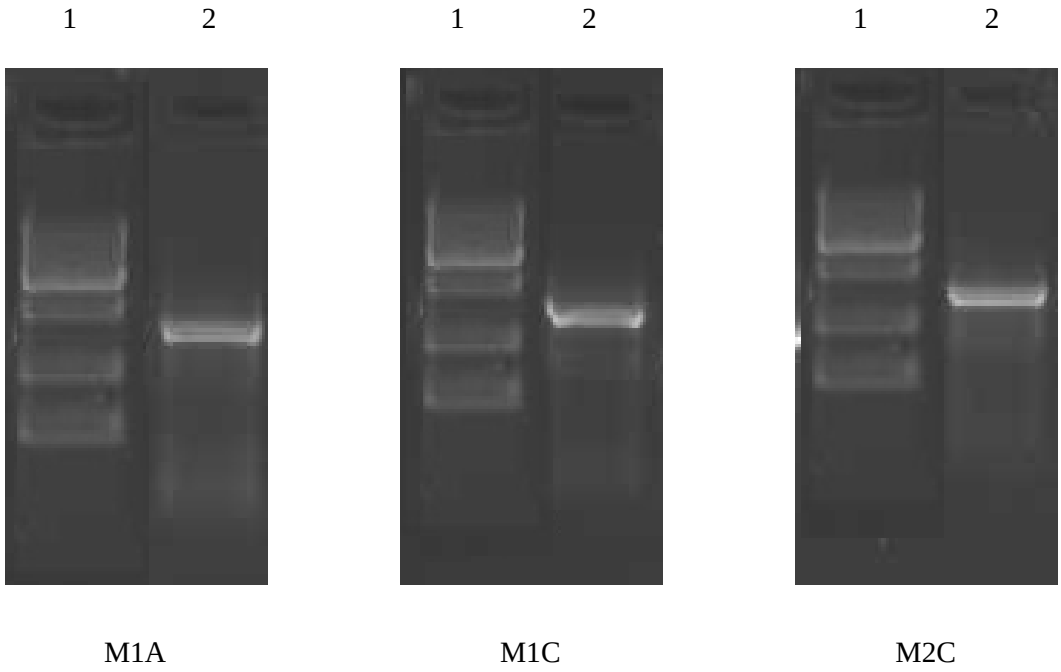


Figure 3.2: 1.2% Agarose gel showing single 1.5 kb and 16S rDNA amplicon. Lane 1: DNA marker (1 kb ladder); Lane 2: 16S rDNA amplicon (1500 bp).

Consensus sequence of **1346bp**, **1302bp and 1348bp** 16S rDNA gene were generated for M1A, M1C and M2C respectively from forward and reverse sequence data using aligner software. The 16S rDNA gene sequence was used to carry out BLAST alignment search tool of NCBI GenBank database. Based on maximum identity score first fifteen sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA 5 software based on 16S rDNA sequence using Neighbor-Joining method.

3.1.7 Dye Decolorization Studies

- **Time Dependent Study of Decolorization of Individual Pure Cultures**

Nutrient medium containing 100 mg/L of all the three model dyes were prepared and transferred to 30 ml culture bottles. The bottles were filled completely and sealed with screw caps to achieve anoxic condition. 1% v/v of 12 hrs old individual pure isolated bacterial strains were then inoculated to every set of bottles and incubated under static condition at 30ºC. The number of bottles in each set corresponded to the number of samples to avoid incorporation of air during repeated sampling. Un-inoculated control was also incubated under same condition to check the abiotic decolorization of the dye. Aliquot of culture media from respective vials were withdrawn at different time intervals and centrifuged at 10,000 rpm for 15 min. Extent of decolorization was monitored by measuring the absorbance of the clear supernatant at the respective absorption maxima of individual dyes (i.e. 635nm for RG-19, 597nm for RNB and 521nm for RR-198). The percentage of decolorization at each time interval was calculated as follows:

$$\%\boldsymbol{Decolorization} = \frac{\boldsymbol{Initial\ absorbance - Observed\ absorbance}}{\boldsymbol{Initial\ absorbance}} \times \mathbf{100} \qquad (3.1)$$

- **Effect of Dye Concentration on Decolorization Efficiency of Isolated Microorganisms**

Dye decolorization capacity of individual organisms was studied with all the three model dyes (RG-19, RNB and RR-198). Efficiency of decolorization was evaluated on the basis of maximum percentage of decolorization achieved at different initial dye concentrations. Nutrient medium containing different concentrations of dye were inoculated with 12 hr

old bacterial cultures. After 24 hr incubation at 30 ºC in static condition, samples were withdrawn and centrifuged at 8000 rpm for 10 min. The cell-free supernatant so obtained was analysed to determine the amount of decolorization. Decolorization of dyes were monitored by measuring the absorbance of the cell free supernatant at their respective absorption maxima using UV-VIS spectrophotometer. Abiotic controls (samples without microorganisms) were included invariably and all the experiments were performed in triplicate. The incubation time was kept constant (at 24 hr) during all decolorization experiments.

- **Effect of Different Physico-Chemical Parameters on Dye Decolorization Efficiency of Isolated Pure Bacterial Strains**

Effect of different physico-chemical factors are found to have tremendous effect on microbial metabolism which in turn affects the decolorization efficiency of microbes. Effects of different process parameters like medium pH (4-11), incubation temperature (20 °C- 45 °C), inoculum volume (1% - 15% v/v), and aeration condition (anaerobic, aerobic & static) on decolorization efficiency of microbes were studied. Nutrient medium containing 100mg/L of dye were incubated at different culture conditions. Decolourization at different culture conditions were studied by changing one-factor-at-a-time keeping the other factors constant. Incubation time was kept constant (i.e. 24hr) for all the experiments. Abiotic controls (samples without microorganisms) were included invariably and all the experiments were performed in triplicate. The percentage of decolorization was calculated as mentioned in Equation 3.1.

3.2 Optimization of dye decolorization efficiency of developed consortia through response surface methodology and kinetic studies

Since, pure bacterial cultures are found to be less efficient (not capable of complete decolorization) or time consuming, use of bacterial consortium may provide more realistic and efficient solution rather than pure culture [25]. Hence trend must be shifted towards the use of bacterial consortium compared to pure cultures.

Process optimization requires thorough evaluation of different parameters affecting the desired output. Conventional single-factor optimization technique is usually time consuming and costly. Apart from that, the combined synergistic effect of various parameters cannot be determined through this exhaustive procedure. Hence, statistical design method including response surface methodology are used to establish a relationship between response and a set of design variables and predicts a model for the response with a minimum number of experiments [190]. This design method could be used as an effective optimization tool to achieve enhanced decolorization of dyes at industrial level.

Several mixed microbial cultures have been reported to be more efficient for color removal from dye effluent as compared to pure cultures [23,25,191,192]. However, comprehensive solutions for azo dyes removal are far from reality, which requires continued search for new organisms and technologies. These findings motivated the rationale for the current study. Since, use of consortium is more realistic and efficient in application rather than pure culture [8,132,135,193]. Hence, our study has been systematically focused on the same to fill the prevailing research gap in the field of bio-decolorization by using a novel developed bacterial consortium consisting of organisms isolated from dye contaminated wastewater for efficient removal of azo-dye from the aquatic systems. Moreover, most municipal WWTPs rely on the microbial component of the activated sludge process (which includes both aerobic and anaerobic treatment process). This study will facilitate application of potential bacterial consortium in existing facilities where segregated colored waste can be targeted for bioremediation.

The first consortium designated as “M12C” comprises of two organisms, i.e. *Bacillus pumillus* (M1C) and *Zobella taiwensis* (M2C). The second consortium (designated as “MA12C”) comprises of three organisms i.e. *Bacillus pumillus* (M1C), *Zobella taiwensis* (M2C) and *Enterococcus durans* (M1A). Dye decolorization capacity of the developed consortia were compared with each other and with the pure strains. Optimization of important process parameters affecting the decolorization efficiency of the two consortia were carried out using RSM based Box-Behnken design. Kinetic parameters for the decolorization of dye were estimated at the optimum conditions using the developed consortia.

3.2.1 Dyes

The textile dye, RG-19 and a mixture of three textile dyes designated as synthetic dye mixture (SDM) were used in this study. A stock solution of RG-19 dye was prepared (10,000 mg/L) and desired concentrations of the dye were obtained by further subsequent dilutions. The SDM was prepared by mixing equal quantity of three dyes (i.e. RG-19, RNB & RR-198) to make the final dye concentration of 10,000 mg/L and the absorbance was recorded at maximum wavelength of 608 nm using UV/visible spectrophotometer. Prepared stock solutions of the model dyes were filtered sterilized using 0.22 µm membrane filter before use.

3.2.2 Culture medium used for decolorization and optimization studies

Decolorization and optimization studies were conducted in 250 mL Erlenmeyer flasks with working volume of 100 mL in synthetic wastewater medium (SWM) with glucose and yeast extract as the carbon and nitrogen sources respectively. The basic composition of SWM was (g/L): $(NH_4)_2SO_4$ 0.28, NH_4Cl 0.23, KH_2PO_4 0.067, $MgSO_47H_2O$ 0.04, $CaCl_22H_2O$ 0.022, $FeCl_36H_2O$ 0.005, NaCl 0.15, $NaHCO_3$ 1.0, and 1 mL/L of a trace element solution containing (g/L); $ZnSO_47H_2O$ 0.01, $MnCl_24H_2O$ 0.1, $CuSO_45H_2O$ 0.392, $CoCl_26H_2O$ 0.248, $NaB_4O_710H_2O$ 0.177, and $NiCl_26H_2O$ 0.02 [125]. Amount of glucose and yeast extract was varied depending upon the results obtained from optimization experiments. The culture media and the glassware were autoclaved at 15 psi (121 $^{\circ}$C) for 20 min prior to the experiments and aseptic conditions were maintained wherever necessary.

3.2.3 Development of Consortium

Different combinations of the isolated bacterial strains were used to develop two novel bacterial consortium. Consortium ‘M12C’ comprises of two bacterial strains i.e. *Bacillus pumillus* (M1C) and *Zobella taiwensis* (M2C) and consortium ‘MA12C’ comprises of all the three isolated bacterial strains i.e. *Bacillus pumillus* (M1C), *Zobella taiwensis* (M2C) and *Enterococcus durans* (M1A). For the development of consortium, a loop full of isolated pure cultures were inoculated separately in nutrient broth and incubated for 6 hr at 30 ºC. Equal volume of 6 hr old cultures were then transferred aseptically into nutrient medium to achieve 5% inoculum in reaction mixture, followed by 24 hr incubation at

30ºC. The consortium thus obtained were used as the source of inoculum for further studies.

3.2.4 Batch Decolorization Experiments Using "M12C" And "MA12C" Bacterial Consortia

Dye decolorization capacity of individual and consortium of organisms was studied with different concentrations of dye (100 mg/L to 1000 mg/L). SWM containing different concentrations of RG-19 dye were inoculated with 12 hr old M1C, M2C, M1A, "M12C" consortium and "MA12C" consortium. After 24 hr incubation at 30 ºC in static condition, samples were withdrawn and centrifuged at 8000 rpm for 10 min. The cell-free supernatant so obtained was analyzed to determine the degree of decolorization. The consortia with comparatively high efficiency were used for further optimization studies of parameters affecting the dye decolorization process.

Before analyzing the synergistic effects of different parameters affecting the process of decolorization, individual effects of these independent variables were studied by varying one factor at a time keeping the other factors constant. Very few microorganisms are capable of using dye as their sole carbon source because of the complex structure of these compounds [194]. Both of the developed consortia M12C and MA12C were unable to use RG-19 dye as the sole carbon source. Effect of different carbon and nitrogen sources on the decolorization efficiency of the developed consortium was studied. Effect of amount of carbon and nitrogen source was studied by adding different concentration of one component and keeping other component constant in minimal salt medium. The effect of incubation temperature (25 °C- 45 °C) and medium pH (5-10) on dye decolorization efficiency of the developed consortia was also analyzed. Dye decolorization potential of M12C consortium was studied taking RG-19 as the model dye. SDM has been used as the model pollutant for studying dye decolorization activity of MA12C consortium. Decolorization of RG-19 and SDM were monitored by measuring the absorbance of the cell free supernatant at their respective absorption maxima (i.e. 635 nm for RG-19 and 608 nm for SDM) using UV-VIS spectrophotometer [195]. Two different types of controls were prepared for the bio-decolorization experiments; the abiotic and the CN- control without glucose and yeast extract. The abiotic control were made with media containing dye without the presence of microorganism and are used to determine the

abiotic loss of dye under different environmental conditions. The CN-control without glucose and yeast extract contained SWM with consortium and the dye were added to test for carbon and nitrogen prerequisite for decolorization. The percentage of decolorization was calculated as mentioned in Equation 3.1.

Further optimization of selected variables was carried out using RSM to examine the linear and synergistic effect of selected parameters.

3.2.5 Optimization using Response surface methodology and Box-Behnken design

Response surface methodology (RSM) is an arrangement of mathematical and statistical techniques used for modelling and analysis of a process which is influenced by numerous variables. The conventional one factor at a time design of process optimization, is laborious, time consuming and incomplete [196]. RSM involves full factorial search by examining simultaneously and in a systematic way all the efficient variation of important components and predicts a model to identify the possible effect of interactions, higher order effects and determine the operational conditions for maximum desired response [197]. The generalized steps considered for optimization of the process parameters using RSM is shown in Figure 3.3.

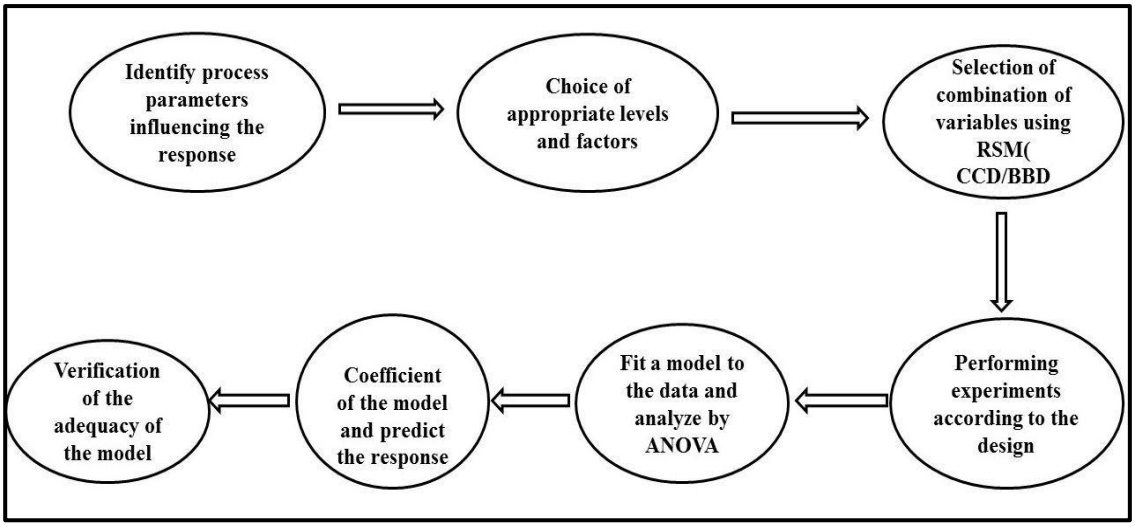


Figure 3.3: Sequential steps for optimization through Response surface methodology

Box-Behnken designs are a class of rotatable or nearly rotatable second-order designs based on three-level incomplete factorial designs [198]. In the present study, important

process parameters influencing the decolorization efficiency of the developed consortia were chosen from batch decolorization studies using one-factor-optimization technique as describes in section 3.2.4. RSM using the three factorial Box-Behnken experimental design was applied to study the synergistic effect of important process variables (incubation temperature, pH, concentration of yeast extract (YE)) for efficient decolorization of RG-19 dye and synthetic dye mixture (SDM) using consortia "M12C" and "MA12C" respectively. Linear and second order polynomials were fitted to the experimental data to obtain the regression equation. The Box-Behnken design is appropriate for the assessment of quadratic response surfaces and generates a second degree polynomial model with comparatively small number of experimental runs which in turn could be used in optimizing a process.

This design requires an experimental number of runs according to:

$$\boldsymbol{N= K^2+K+C_p} \tag{3.2}$$

Where K is the factor number which is 3 in this case and C_p is the number of replications at the center point which has been set at 5 in the present study. The design which was developed using Design-expert software (Stat-Ease, trial version 9.0.4.1) resulted in 17 experimental runs. These experimental runs were randomized to maximize the effects of unexplained variability in the observed responses due to irrelevant and pertinent factors. Table 3.2 and Table 3.3 shows the experimental parameters and the experimental Box–Behnken design levels used for the optimization study of "M12C" and "MA12C" consortium respectively, which were selected based on preliminary experiments. The relation between the coded and actual values is described as follows:

$$\boldsymbol{X_c = \frac{X_a - X_o}{\Delta X_f}} \tag{3.3}$$

Where X_c and X_a are the coded and actual values of the independent variable respectively. X_o is the actual value of the independent variable at the center point, and ΔX_f is the step change of X_a. A second degree polynomial was fitted to the experimental data to estimate the response of the dependent variable and predict the optimal point. Considering all the

linear terms, square terms and linear interaction items, the quadratic response model can be described as:

$$\boldsymbol{Y = b_o + b_1 A + b_2 B + b_3 C + b_{12} AB + b_{13} BC + b_{23} AC + b_{11} A^2 + b_{22} B^2 + b_{33} C^2} \quad (3.4)$$

where Y is predicted response, A, B and C are independent variables, b_0 is offset term, b_1, b_2, b_3 are linear effects and b_{11}, b_{22}, b_{33} are squared terms and b_{12}, b_{13}, b_{23} interaction terms.

Table 3.2: Experimental design levels of the chosen variables for Box-Behnken design for "M12C" consortium

Independent variables	**Symbols**	**Coded and Actual Levels**		
Coded level		Low (-1)	Middle(0)	High (+1)
Temperature (ºC)	A	25	35	45
pH	B	5	7.5	10
Yeast Extract Concentration (g/100ml)	C	0	0.75	1.5

Table 3.3: Experimental design levels of the chosen variables for Box-Behnken design for "MA12C" consortium

Independent variables	**Symbols**	**Coded and Actual Levels**		
Coded level		Low (-1)	Middle (0)	High (+1)
Temperature (ºC)	A	20	32.5	45
pH	B	5	7.5	10
Yeast Extract Concentration (g/100ml)	C	0.1	1.3	2.5

3.2.6 Study of Biodegradation Kinetics

To understand how a system functions, we need a kinetic description of the system and its parameters. Study of decolorization kinetics is necessary for scaling up the biological treatment methods to an industrially relevant scale. In chemical kinetics, the order of

reaction with respect to a given substance is defined by the exponent to which its concentration term is raised in the rate equation. Dye decolorization kinetics using biological treatment method commonly found to follow First-order reaction with respect to dye concentration [119,195,199]. The first-order rate constants (k) for dye decolorization reactions can be estimated using the integrated first-order rate law:

$$\mathbf{ln}[\boldsymbol{C_t}] = (-)\boldsymbol{kt} + \mathbf{ln}[\boldsymbol{C_0}] \quad (3.5)$$

Where, C_0 is the initial dye concentration and C_t is the dye concentration after time 't'.

Hence, the slope of the linear plot between $\ln[C_t/C_0]$ against time (t) gives the value of the rate constant for the decolorization reaction for a particular dye concentration.

For the system involving living cells and/or enzymes, Michaelis-Menten type rate model is widely used to determine the kinetic parameters [84,195] and is described by the equation:

$$\boldsymbol{V_0} = -\frac{\boldsymbol{d[Substrate]}}{\boldsymbol{dt}} = \frac{\boldsymbol{V_{max}[Substrate]}}{\boldsymbol{[Substrate]+K_m}} \quad (3.6)$$

where, V_0 is the substrate consumption rate (mg L^{-1} hr^{-1}); V_{max} is the maximum substrate consumption rate (h^{-1}) and K_m is the Michaelis-Menten constant [195].

The values of V_{max} and K_m are transformed by a double reciprocal approach to Lineweaver-Burk equation as follows:

$$\frac{\mathbf{1}}{\boldsymbol{V_0}} = \frac{\boldsymbol{K_m}}{\boldsymbol{V_{max}S}} + \frac{\mathbf{1}}{\boldsymbol{V_{max}}} \quad (3.7)$$

Hence, the value of K_m and V_{max} could be calculated from the linear plot between $1/[V_0]$ versus 1/[S] with a slope of K_m/V_{max} and intercept of $1/V_{max}$.

To understand how the system functions with increasing dye concentration and for determining maximum decolorization rate of RG-19 dye using M12C consortium and SDM using MA12C consortium, we used Michaelis-Menten kinetic model.

Concentration of dye in the industrial effluent greatly influences the decolorization efficiency of microorganisms. Dye house effluents are reported to have dye concentration in the range of 10 mg/L to 250 mg/L [200]. A wide range of dye concentration ranging from 25 mg/L to 1500 mg/L has been selected based on the above facts to study its effect on decolorization kinetics. Medium containing different concentrations of dye were

inoculated with 1% (v/v) inoculum and incubated under optimized conditions. Samples were withdrawn at regular time interval to analyse residual dye concentration. Control flask containing 25 mg/L of dye without inoculum were incubated at same condition to check abiotic loss of dye. All the experiments were performed in triplicate.

3.2.7 Analytical Methods to Study Biodegradation of Dyes

To confirm the process of biodegradation, dye containing medium before and after decolorization were analyzed using different analytical techniques such as UV-Vis spectrophotometer, HPLC, FTIR and GC-MS analysis.

- **UV-Vis Spectrophotometer**

Different dyes absorb light at a particular wavelength in visible region which depends upon the type of chromophore attached to the dye molecule. Biodegradation results in breakdown of the large molecular structure of dye molecule which leads to decrease in absorption peaks in the visible region. To monitor the process of biodegradation of our target azo dyes through UV-Vis spectroscopy, dye decolorized medium was collected before and after decolorization and centrifuged at 10,000 rpm for 10 min. The supernatant so obtained was filtered through 0.22 µm syringe filter and was analyzed using UV-Vis spectrophotometer (UV-VIS spectrophotometer-3200, LabIndia Instruments Pvt Ltd, India) to study the difference in absorbance peak.

- **HPLC, FTIR and GC-MS Analysis**

After complete decolorization, 40 ml of the degraded sample was centrifuged at 10,000 rpm for 10 min and the supernatant was collected. Then the cell free supernatant was extracted using ethyl acetate in 1:1 proportion and the extracted sample was evaporated to dryness using rotary evaporator. The dried residue was subsequently dissolved in HPLC grade methanol and used for HPLC, FTIR and GC-MS analysis.

HPLC unit equipped with (MD-2015 plus, Jasco, Japan) UV-VIS detector was used to study the degradation of RG-19 dye. Samples were eluted in C18 column (5µm, 4.6 mm×250 mm) with mobile phase (60%) methanol, (38%) water and (2%) acetic acid at flow rate of 1.0 ml min^{-1}.

FTIR analysis was required to study the changes in surface functional groups of the dye containing medium before and after decolorization process. Analysis was carried out using (Nicolet^{TM} iS^{TM}10, Thermofisher Scientific, USA) and changes in terms of % transmission were observed at different wavelengths spectrophotometer in the mid-infrared region of 400-4000cm^{-1} with 16-scan speed.

GC-MS analysis of the samples were performed through Agilent 7890-B Gas Chromatography coupled with 5977-A Mass Spectrometry to identify the dye degradation metabolites. The analysis was performed in the temperature programming mode at an ionization voltage of 70eV. The flow rate of helium was 1.0 ml min^{-1}. The oven temperature range was programmed from 80 °C to 280 °C at a rate of 10 °C min^{-1}, and held for 10 min. The highest resolution chromatographic peaks were scanned to obtain their corresponding mass fragmentation and were characterized based on similarity between their mass spectrum and those presented by NIST library.

3.3 Complete degradation of Remazol navy blue in an integrated MFC-Aerobic two stage system and simultaneous bioelectricity generation using the developed consortium “MA12C”

Biotransformed products resulted from anaerobic decolorization of azo dyes are known to be recalcitrant to further degradation in the same condition and needs to be treated further aerobically. In recent years, degradation of azo dyes in sequential anaerobic-aerobic process is gaining attention due to its ability to completely mineralize the complex dye molecule into simple nontoxic intermediates without the accumulation of toxic aromatic amines. However, the kinetics of anaerobic decolorization is very slow which represents a limiting factor in such two-step approach of azo dye treatment.

Anaerobic environment and exo-electrogenic nature of microorganisms utilised in MFC anode help in reductive degradation of azo dyes. In addition to that, aerated cathode as the final electron acceptor of MFCs speed up the microbial metabolism and kinetics of azo dye degradation is expected to be faster as compared to traditional anaerobic systems.

The objective of the present study is to investigate the feasibility of utilizing bacterial strains isolated from dye contaminated wastewater for complete degradation of azo dye in an MFC coupled with aerobic reactor system and simultaneous production of bioelectricity. Initial studies were carried out using the consortium "MA12C" under the optimum conditions to compare the decolorization of different azo dyes both in static and MFC mode and simultaneous production of bio-electricity. The second part of the study has been focused on complete degradation of a commonly used azo dye Remazol navy blue (RNB) in an integrated microbial fuel cell coupled with aerobic system. Kinetics of decolorization of RNB by the developed consortium in MFC system was compared with that of decolorization kinetic in static condition. The effects of initial dye concentration and external resistance on MFCs performance in terms of RNB decolorization and power generation were evaluated. Dye degradation metabolites from each stage of the integrated MFC-aerobic system has been studied and compared. Phytotoxicity analysis has been carried out to evaluate the toxic effect of the effluents released from each stage of the two step process.

3.3.1 Dye and Chemicals

Textile azo dyes RG-19, RNB, RR-198 and synthetic dye mixture (SDM) containing equal quantity of all the three dyes has been used for the preliminary investigations in this study to compare the dye decolorization potential of "MA12C" strain both in static and MFC mode. In the second part of the investigation, RNB has been used as the model dye to study its complete mineralization in a sequential anaerobic-aerobic system. All the media components used for bacterial culture and chemicals used for analytical methods were purchased from Himedia and are of analytical grade.

3.3.2 Inoculation and Electrolyte Conditions

Each of the MFCs were inoculated with 1 mL of 12hr old actively growing "MA12C" consortium. The anolyte medium composition was same as the SWM used in the previous study and as explained in section 3.2.2. However only yeast extract (1.888 g/100mL) has been used as the co-substrate and pH of the medium was kept 8.29 in this study as concluded from the optimization study involving MA12C consortium. Cathode

compartment was filled with 50 mM phosphate buffer (21mM - NaH_2PO_4 and 29mM-Na_2HPO_4), pH 7.0 in all the experiments.

3.3.3 The Integrated MFC-Aerobic Reactor System Assembly and Operation

The H-type MFCs were constructed using two identical plastic bottles (capacity 1000 mL). The anode and cathode compartments were separated by a proton exchange membrane (Nafion 117). The electrodes were constructed from carbon cloths with same projected surface area of 20 cm^2. The electrodes were connected through insulated copper wire and the exposed connections were coated with nonconductive epoxy for insulation. An external load of 1000 Ω was applied in all experiments except when different resistors (22 Ω - 1 MΩ) were used to determine the power generation capacity of MFCs as a function of load and the potential across the resistor was recorded using a digital multimeter (DT830D).

The anolyte medium was sterilised through autoclaving at 121 °C for 15 min before use in anode compartment. Anode compartments were surface sterilised with 100% pure ethanol followed by 30 min exposure to UV light. All the experiments were carried out in batch mode with a load of 1000 Ω at room temperature (25 °C -35 °C) with working volume of 1000 mL. During start-up, the MFCs were operated in fed-batch mode till stable voltage across the 1000 Ω resistor has been reached in order to achieve reproducible MFC performance in all replicate MFC systems. Each fed-batch cycle started when the voltage across the two electrodes falls below 50 mV and in the beginning of each cycle 75% of the anode contents were replaced with fresh medium. After four consecutive fed-batch cycles, a stable voltage was obtained across 1000 Ω resistor which indicates the colonization of bacteria on the electrode surface. Figure 3.5b and 3.5c show the FE-SEM images of carbon cloth electrodes before and after development of biofilm respectively. The catholyte was continuously aerated at a flow rate of 100 mL air/min using an aquarium pump. Prior to inoculation the anodic chamber was sparged with nitrogen gas for 10 min and the MFC headspace was also filled with nitrogen gas to maintain the anaerobic environment. MFCs with dye containing synthetic medium and without microorganisms has been used as control throughout the studies.

At the end of each batch cycle, the effluent from MFC stage was fed-into the subsequent aerobic chamber for second stage treatment. Schematic diagram of the integrated MFC-aerobic system is shown in Figure 3.4 and the experimental setup is shown in Figure 3.5a.

The spent medium and the microbial consortium washed out from the MFC stage has been used for treatment of the dye degradation metabolites in the subsequent aerobic stage. No other exogenous inoculum source was used for the aerobic stage operation. Working volume of the aeration chamber was 1000 mL. The chamber was continuously sparged with air at a flow rate of 200 mL/min using a ceramic air sparger and an aquarium pump.

For proper mixing, the content of aerobic chamber was continuously agitated using a magnetic mixture. The system was operated at ambient temperature without any temperature control system. The laboratory temperature varied from 25 ˚C (night time) to 35 ˚C (day time) during the operation.

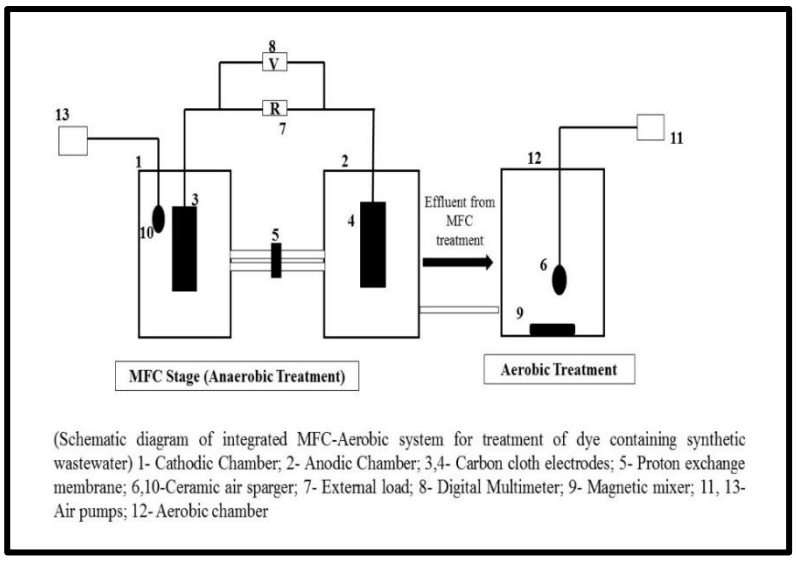


Figure 3.4: Schematic diagram of the experimental set-up of integrated MFC-aerobic system

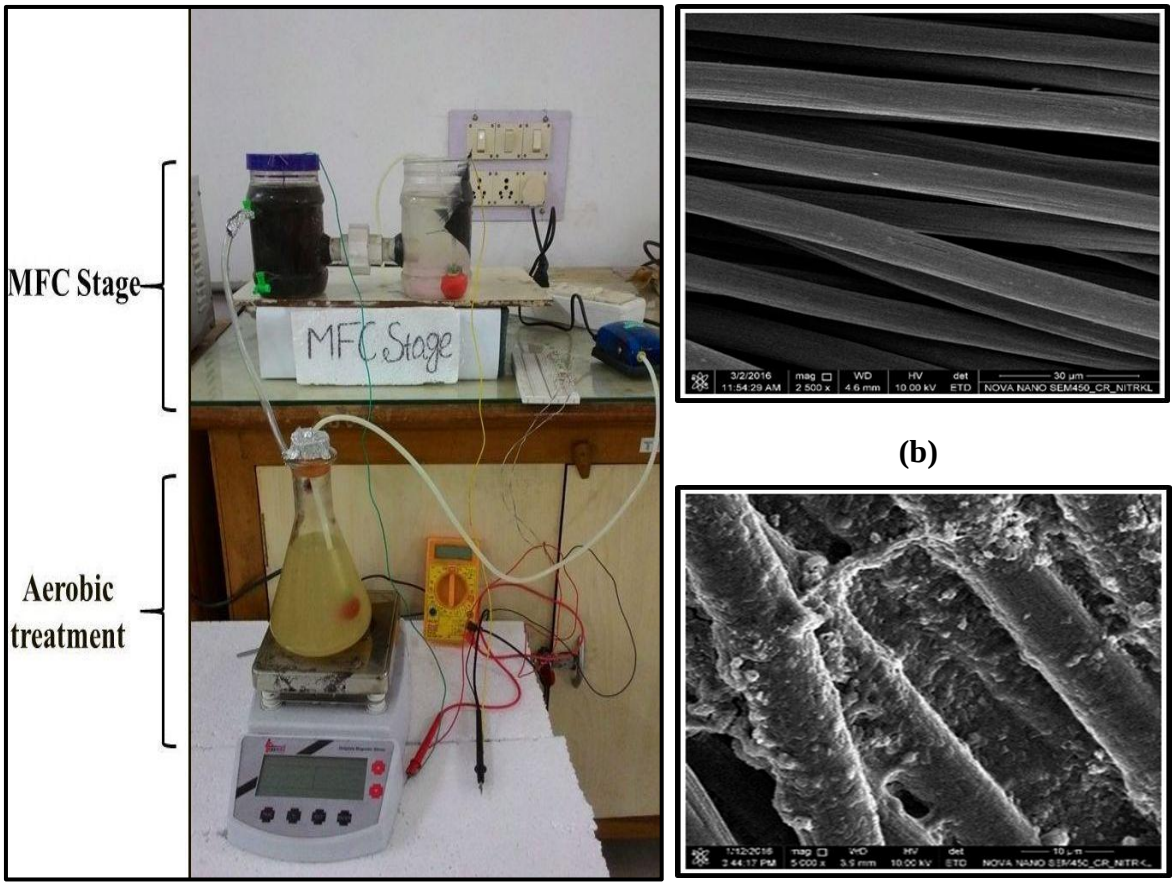


Figure 3.5: (a) Experimental set-up of Integrated MFC-aerobic system; (b) FE-SEM images of anode electrode before biofilm development; (c) FE-SEM image of Anode electrode after biofilm development

3.3.4 Dye Decolorization and Kinetic Studies of "MA12C" Consortium in MFC System

Decolorization of all the three model dyes and the synthetic dye mixture (SDM) was monitored by UV-VIS spectroscopy at their respective absorption maxima. The dye decolorization efficiency of the developed consortium was calculated in terms of % decolorization as mentioned in Equation 3.1.

The dye decolorization kinetics using the developed consortium in MFC was modelled using first-order kinetic models. The first-order decolorization kinetic constants (k) can be estimated using the integrated first-order rate law as mentioned in Equation 3.5.

The effect of initial dye concentration on kinetics of RNB decolorization using "MA12C" consortium was studied in MFC system at RNB concentrations ranging from 50 mg/L to 1000 mg/L. Effect of different MFC external resistance on decolorization kinetics of RNB was determined at 100 mg/L RNB concentration.

3.3.5 Analysis of Electrochemical Parameters

The potential difference across the two electrodes was monitored using a digital multimeter. When the MFC attained a stable voltage across 1 kΩ resistor, the circuit was connected to various external resistances ranging from 22Ω to 1MΩ to construct the polarization curve.

Current (I) was calculated at a resistance (R) from the voltage (V) using the Ohm's law: I = V/R. Power (P) was calculated as P = VI. The current density and power density values were calculated by normalizing current and power values to the projected surface area of the anode (20 cm^2).

3.3.6 Study of Dye Degradation Products

Biodegradation of RNB was confirmed through UV-VIS, FTIR and GC-MS analysis. Completely decolorized medium was centrifuged at 10,000 rpm for 10min to remove the biomass. Supernatant so obtained was filtered through 0.22 µm syringe filter and directly used for UV-VIS analysis. 40 mL of the cell free supernatant was extracted using ethyl acetate in 1:1 proportion and evaporated the extracted sample to dryness using rotary evaporator. The dried residue was then dissolved in HPLC grade methanol and used for FTIR and GC-MS analysis. The changes in surface functional groups of RNB dye before and after treatment was investigated through FTIR analysis. FTIR analysis was carried out using (Nicolet^{TM} iS^{TM}10, Thermofisher Scientific, USA) spectrophotometer in the mid-infrared region of 400-4000 cm^{-1} with 16-scan speed. GC-MS analysis was performed for identification of metabolites formed after each stage treatment of RNB dye in the integrated MFC-aerobic sequential process. GC-MS analysis of the samples were performed through Agilent 7890-B Gas Chromatography coupled with 5977-A Mass Spectrometry. The analysis was performed in the temperature programming mode at an ionization voltage of 70 eV. The flow rate of helium was 1.0 ml/min. The oven temperature range was programmed from 80 °C to 280 °C at a rate of 10 °C min^{-1}, and

held for 10 min. The highest resolution chromatographic peaks were scanned to find their corresponding mass fragmentation and were characterized based on similarity between their mass spectrum and those presented by NIST library.

3.3.7 Toxicity Analysis Through Phytotoxicity Study

As the treated wastewater is mostly used for agriculture purpose [81], toxicity of treated and untreated effluent had direct impact on fertility of soil, plant growth and agricultural yield. Phytotoxicity study has been found to be a well-practiced method to assess toxic effect of untreated and treated dye containing effluent [25,125,201]. Phytotoxicity study has been carried out with a common Indian agricultural crop *Phaseolus mungo* to study the toxic effect of RNB dye before and after treatment in the integrated MFC-aerobic two stage system. The extracted metabolites from each stage of the integrated anaerobic-aerobic system was dissolved in 10 mL sterile distilled water to make the final concentration of 300 mg/L. Ten healthy seeds of P. mungo were sowed in plastic sand pot. The sand was washed properly and oven dried before being used in sand pots. The seeds were incubated at room temperature by regular watering with RNB solution (300 mg/L) and RNB degradation metabolite solutions (300 mg/L). A set of 10 seeds supplied with distilled water were used as control. Seeds were supplied with equal volume (10 mL) of water, dye solution and metabolite solution per day. After seven days of incubation, percentage germination, root length and shoot length of the germinated seeds were recorded to assess the phytotoxic effect of the dye solution and its degradation metabolites. All the studies were carried out in triplicate. Percentage germination was calculated as follows [201]:

$$\%\boldsymbol{Germination} = \frac{\boldsymbol{Number\ of\ seeds\ germinated}}{\boldsymbol{Number\ of\ seeds\ sowed}} \times \mathbf{100} \qquad (3.8)$$

Chapter 4

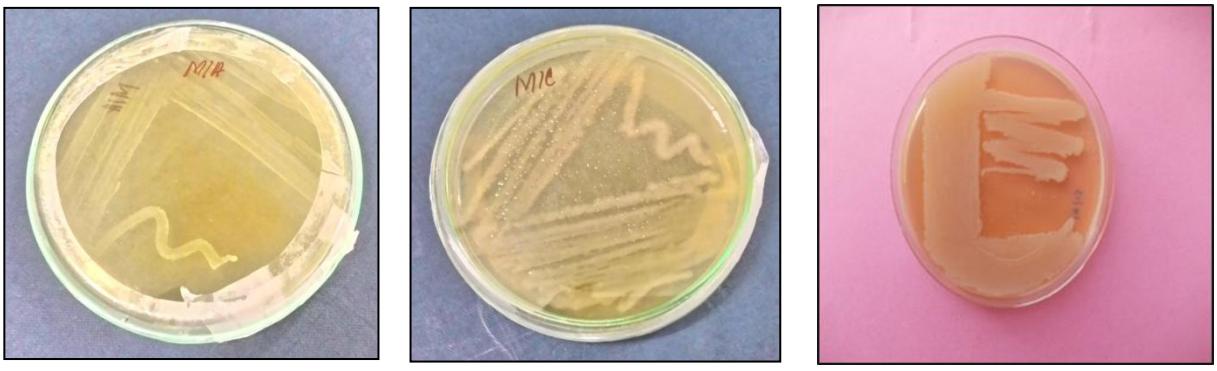
Results and Discussion

4.1 Isolation and Screening of Potent Dye Decolorizing Bacterial Strains and Study of Their Dye Decolourization Capacity

4.1.1 Screening and Isolation of Dye Decolorizing Bacterial Strains

Several bacterial colonies showing zone of decolorization were observed on the dye containing agar plates after 48 hr of incubation at 30 °C. Fifty morphologically distinct colonies were selected for further purification and eleven pure bacterial cultures were isolated after final isolation process. A number of researchers considered textile industry effluent as an efficient source of potent dye decolorizing bacteria due to the natural adaptation to the higher dye concentration and its toxic effect [193,202–205].

Dye decolorization efficiency of the selected eleven isolates were studied in dye containing liquid medium with all the three model dyes, however highest decolorization was noticed with RNB. Dye decolorization efficiency of the isolates for decolorization of RNB is shown in Table 4.1. All the bacterial isolates showed potential to decolorize dye containing nutrient medium, however their decolorization efficiency varied substantially.



(M1A) **(M1C)** **(M2C)**

Figure 4.1: Isolated pure cultures

Out of these eleven strains, three strains showing highest decolorization and tolerance to dye mixture were selected and were designated as M1A, M1C, and M2C (Figure 4.1). Further studies were carried out using these three isolated pure bacterial strains.

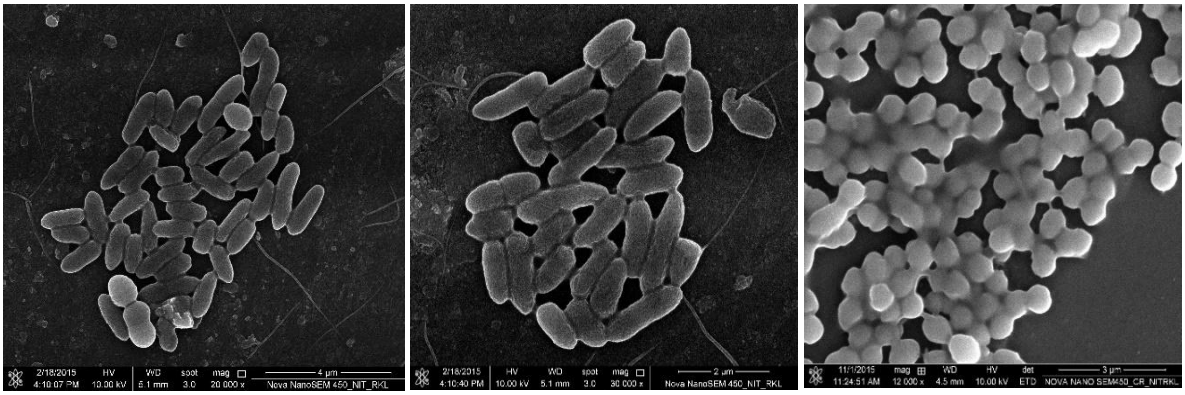
Table 4.1: Decolorization efficiency of all the isolated strains for RNB decolorization

Isolated bacterial strains	Maximum % Decolourization achieved
M1A	**94%**
M1B	65%
M1C	**93%**
M2A	55%
M2B	43%
M2C	**96%**
C1	36%
C2	60%
S1	29%
S2	34%
S3	50%

4.1.2 Identification of Isolated Microorganisms

The morphological characteristics of the isolated organisms were observed by FE-SEM analysis. Gluteraldehyde fixation method has been used to fix the isolates on to a glass slide followed by subsequent drying with increasing concentration of ethanol. Samples were coated with a thin layer of gold to make the surface more efficient for electron scattering. The images obtained from FE-SEM analysis confirms that the isolates are of bacterial origin. M1A is round shape whereas M1C and M2C were of rod shape (Figure 4.2).

Through 16s rDNA analysis, on quality evaluation of all isolated DNA, a single band of high-molecular weight DNA has been observed on 1.2% agarose Gel. The fragment of 16S rDNA gene was amplified by PCR using 8F and 1492R from isolated DNA. A single discrete PCR amplicon band of 1500 bp was observed (Figure 3.2). The PCR amplicon was purified and further processed for the sequencing. Forward and Reverse DNA sequencing reaction of PCR amplicon was carried out with **8F** and **1492R** primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequence of **1346bp**, **1302bp and 1348bp** 16S rDNA gene were generated for M1A, M1C and M2C respectively from forward and reverse sequence data using aligner software. All positions containing gaps and missing data were eliminated. The 16S rDNA partial sequences of M1A, M1C and M2C have been deposited in GenBank under accession number KC213474.1, FJ999669.1 and KJ741252.1 respectively. Based on nucleotide homology and phylogenetic analysis, M1A, M1C and M2C strains were identified as *Enterococcus durans* GM13, *Zobellella taiwanensis* AT1-3 and *Bacillus pumilus* HKG212 respectively. The phylogenetic tree explained the evolutionary relationship of the isolates with other biological species based upon the similarities and differences in their physical and/or genetic characteristics. The taxa joined together in the tree are implied to have descended from a common ancestor. Unrooted trees illustrate the relatedness of the leaf nodes without making assumptions about ancestry at all. Figure 4.3, Figure 4.4 & Figure 4.5 shows the phylogenetic trees of M1A, M1C and M2C respectively with the 15 most similar sequences. Details about the results of maximum identification score and distance matrix of the isolates are given in Appendix-I.



(a) **(b)** **(c)**

Figure 4.2: FE-SEM images of (a) M1C, (b) M2C, (c) M1A

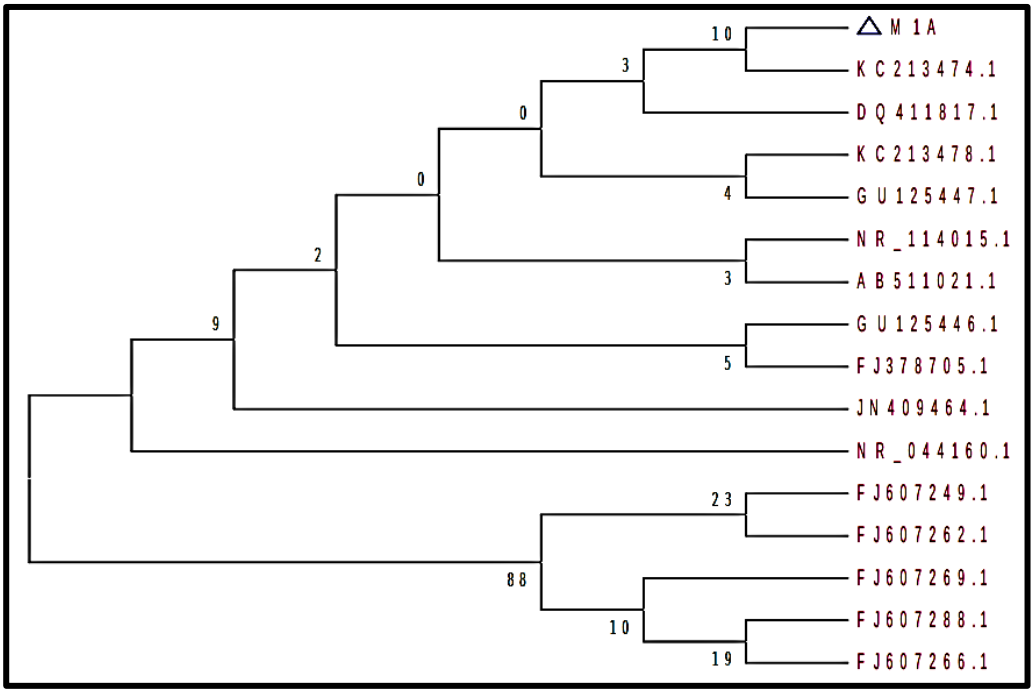


Figure 4.3: Phylogenetic tree showing evolutionary relationship of isolate 'M1A' with 11 taxa using Neighbor-Joining method.

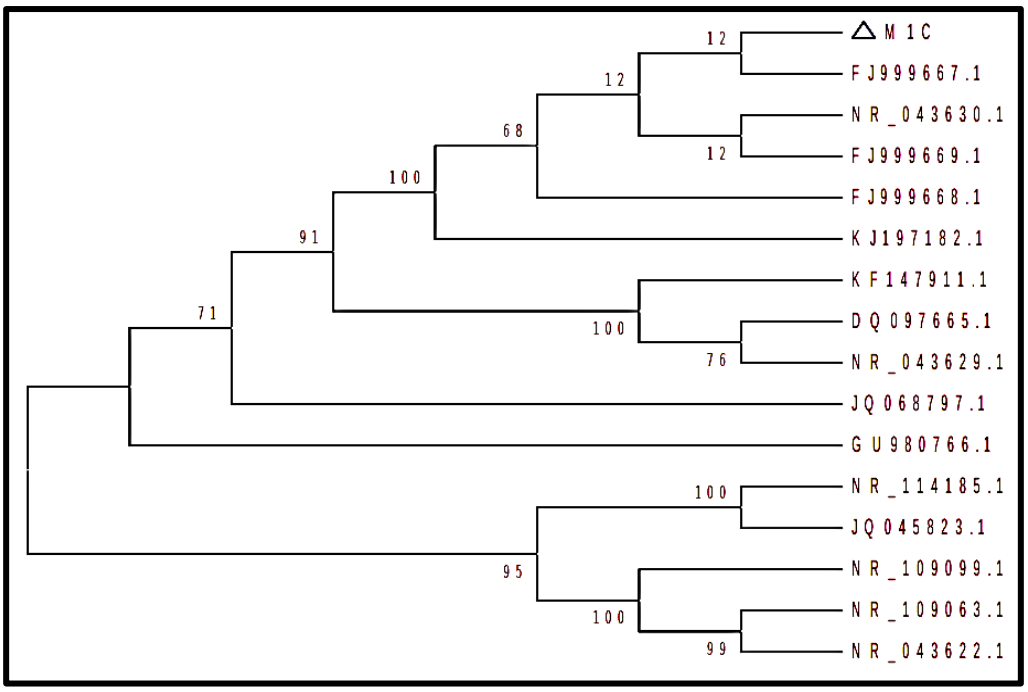


Figure 4.4: Phylogenetic tree showing evolutionary relationship of isolate 'M1C' with 11 taxa using Neighbor-Joining method.

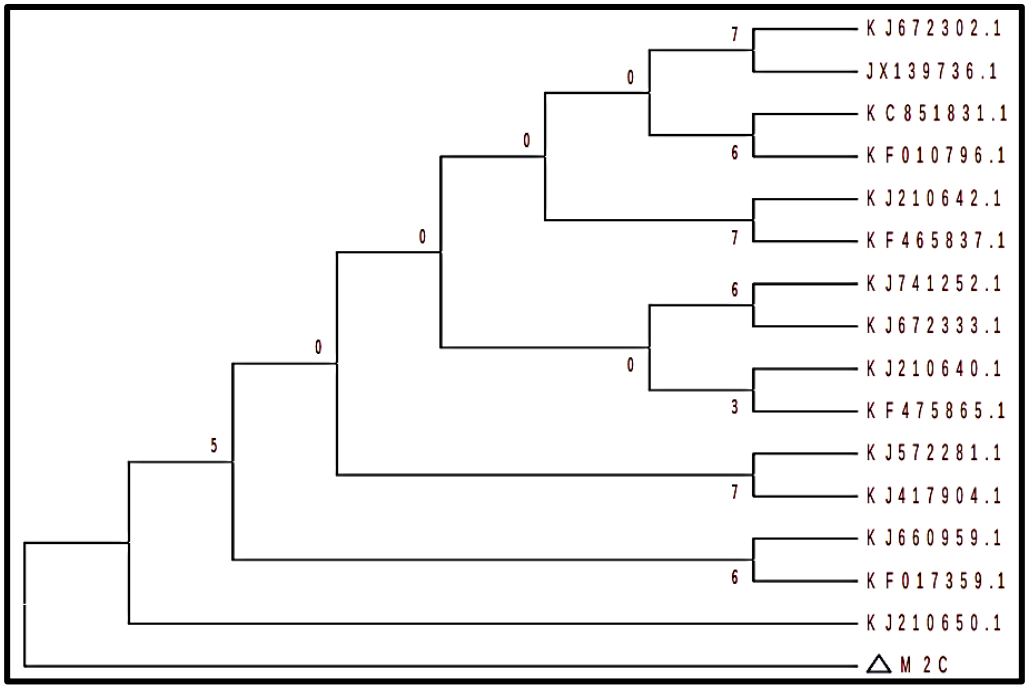


Figure 4.5: Phylogenetic tree showing evolutionary relationship of isolate 'M2C' with 11 taxa using Neighbor-Joining method.

4.1.3 Dye Decolorization Studies

Decolorization capacity of isolated pure strains was studied under different environmental conditions. Batch decolorization studies have been carried out using all the three isolated strains to evaluate their decolorization efficiency for the decolorization of RG-19, RNB and RR-198 under different physico-chemical conditions.

- **Effect of Incubation Period on Decolorization Efficiency of Isolated Organisms**

Decolourization of all the three model dyes by the isolated bacterial strains was recorded at different time intervals (figure 4.6a, 4.6b, 4.6c). The results showed that, maximum decolorization of all the three model dyes was achieved within 30 hr of incubation using all the three isolated strains. A rapid increase in color removal was noticed up to 24 hr of incubation, however after 24 hr the progress in color removal became slow and the results recorded was negligible. Similar finding was reported by Alalewi et. al (2012) for decolorization of two textile dyes i.e Acid Orange 7 and Direct blue 75 using two isolated bacterial species where maximum decolorization was achieved within 24 hr of incubation [206]. M2C was found to be capable of achieving 89% decolorization for RG-19, 95% decolorization for RNB and 94% decolorization for RR-198 dye within 24hr of

incubation. M1C and M1A showed a reduced amount of decolorization in comparison to M2C. Using M1C 85% RG-19, 90% RNB and 89% RR-198 decolorization was achieved, however M1A showed 86% RG-19, 91% RNB and 92% RR-198 decolorization within 24hr of incubation. It could be inferred from Figure 4.6d that, under the similar conditions, decolorization increased with increase in bacterial growth. Increase in biomass growth with a concomitant increase in dye decolorization suggests that, the dye decolorization activities of the isolated strains are associated with their corresponding cell growth which agrees with findings of other researchers [53,207].

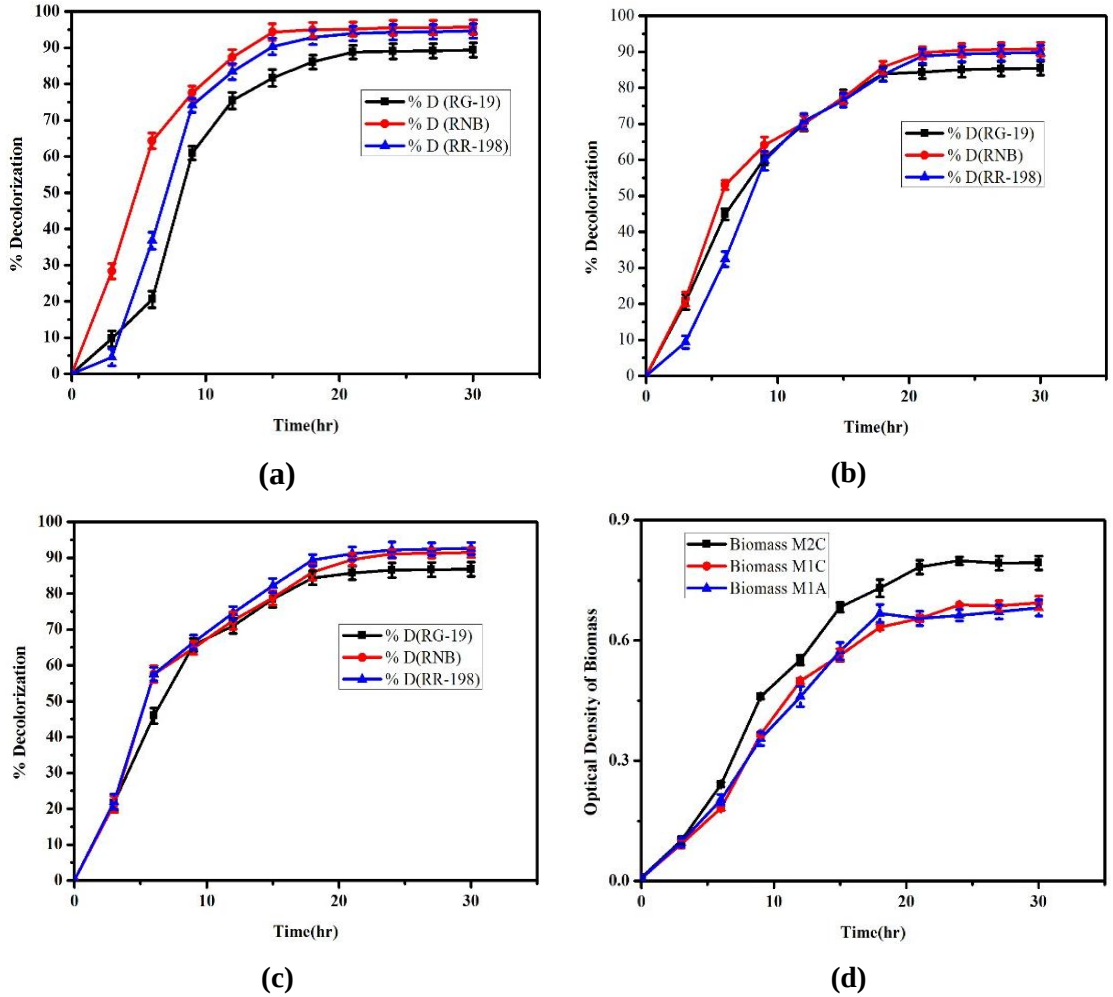


Figure 4.6: Decolorization profile of individual dyes using (a) (M2C) *Bacillus pumilus* HKG212; (b) (M1C) *Zobellella taiwanensis* AT 1-3; (c) (M1A) *Enterococcus durans* GM13; (d) Biomass growth of three isolates with time in presence of 100 mg/L RG-19 dye

Ogugbue et al. (2011) studied biodegradation of Triarylmethane dyes using *Aeromonas hydrophilia*, and observed that the increase in color removal is directly affected by the

increase in biomass growth with time. Similar observation was also reported by Asad et al. (2007) for the decolorization of Remazol Black B using three bacterial strains isolated from textile effluent where color removal increased with corresponding increase in biomass growth. As all the three strains achieved their maximum decolorization efficiency within 24hr of incubation, further studies were done taking this time frame into consideration.

- **Effect of dye concentration on decolorization efficiency of microorganisms**

Concentration of dye is an important parameter which can influence the decolorization efficiency of an organism. Higher dye concentrations can be toxic to microorganisms besides lower dye concentration may affect the ability of enzyme to recognize the dye molecule. For industrial application, it is imperative to study the tolerance and decolorization efficiency microorganisms to higher dye dye concentrations. Dye house effluents are reported to have dye concentration in the range of 10 mg/L to 250 mg/L [8]. A wide concentration ranges of all the three model dyes (RG-19, RNB & RR-198) were chosen to study the tolerance level and decolorization capacity of the isolated pure cultures. Figure 4.7a, 4.7b, 4.7c shows the % decolorization of all the three model dyes at different initial concentrations using all the three isolated pure cultures. The isolated organisms were found to be highly tolerant to higher dye concentrations and were able to achieve more than 80% of color removal in a concentration range of 100-1000 mg/L. At initial dye concentration of 100-200 mg/L, M2C showed 89% decolorization for RG-19 whereas 96% and 94% decolorizations were obtained for RNB and RR-198 respectively. As the dye concentration was increased to 1000 mg/L the decolorization efficiency reduced to 85% for RG-19, 80% for RNB and 81% for RR-198. The similar trend was followed by both M1A and M1C where decolorization efficiency was found to be decreased with increase in initial dye concentrations. Figure 4.7d depicts the effect of dye concentration on biomass growth of the three isolates strains. This study clearly shows that, the increase in dye concentration has very little effect on the decolorization efficiency as well as biomass growth of the isolated organisms within 100-600 mg/L dye concentration. However, with further increasing the dye concentration imparts a negative effect on the decolorization efficiency as well as on the biomass growth of the organisms. This can be attributed to the fact that, at higher dye concentrations, metabolic activity of

microbes gets affected by the toxic effects of dye molecules and their degradation metabolites which in turn inhibits growth and hence the decolorization process gets affected [207]. Several authors have also reported the decreasing color removal efficiency of different bacterial strains with increasing dye concentration [208,209].

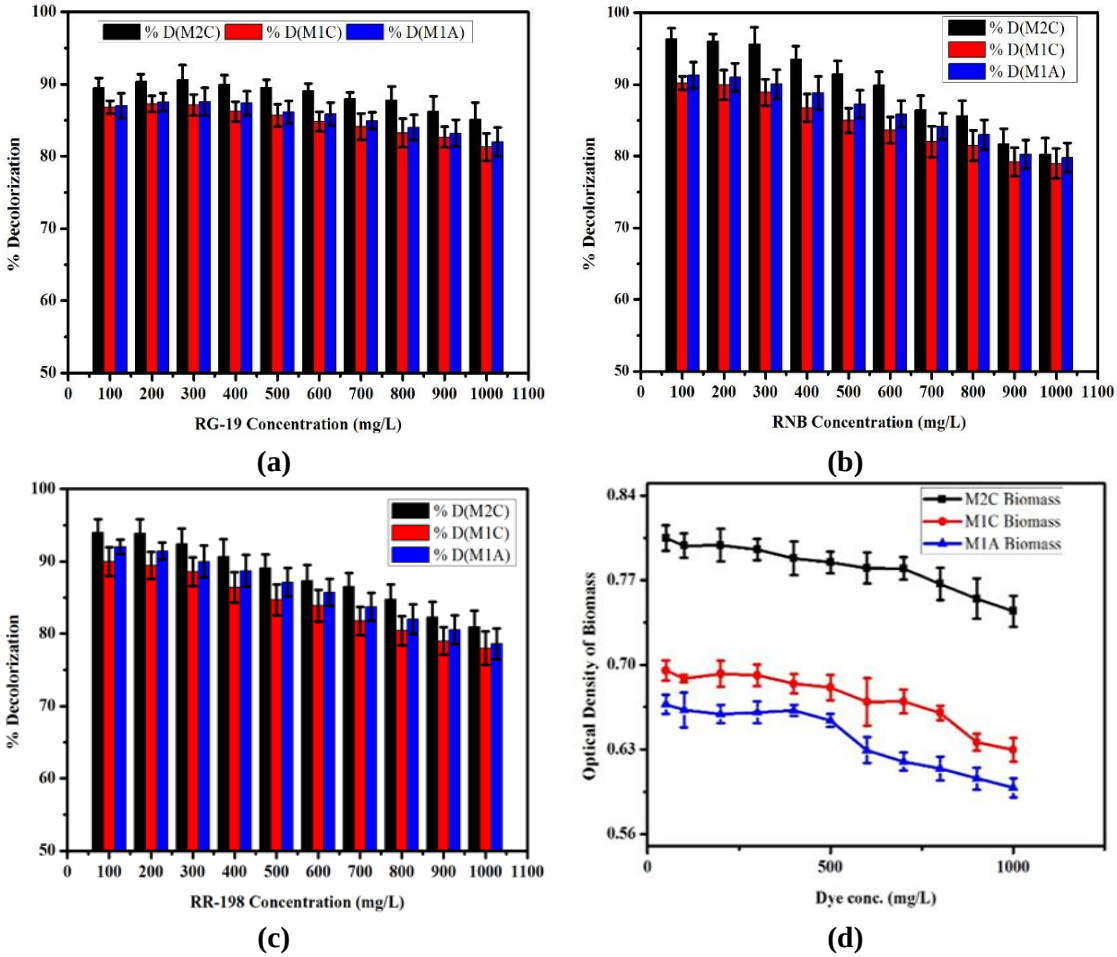


Figure 4.7: Effect of dye concentration on decolorization efficiency of individual strains (a) for RG-19; (b) for RNB; (c) for RR-198; (d) Effect of RG-19 concentration on biomass growth of individual strains

- **Effect of Different Physico-Chemical Parameters on Dye Decolorization Efficiency of Isolated Pure Bacterial Strains**

For any kind of bioremediation process, physico-chemical parameters like aeration condition, inoculum volume, medium pH, incubation temperature etc. are considered as vital components that affect the overall efficiency of the process [210–213]. Study of

individual effects of each parameter is essential before analysing the synergistic effects of these factors.

- **Effect of Inoculum Volume**

In batch process where organisms grow in a fixed amount of nutrient, inoculum volume becomes an important factor affecting the overall efficiency of the process. Small inoculum volume slows down the overall rate of biological reaction which leads to decrease in rate of dye decolorization. However, inoculum volume larger than the optimum value results in early depletion of nutrient and microorganisms attain the death phase which leads to decrease in dye decolorization rate.

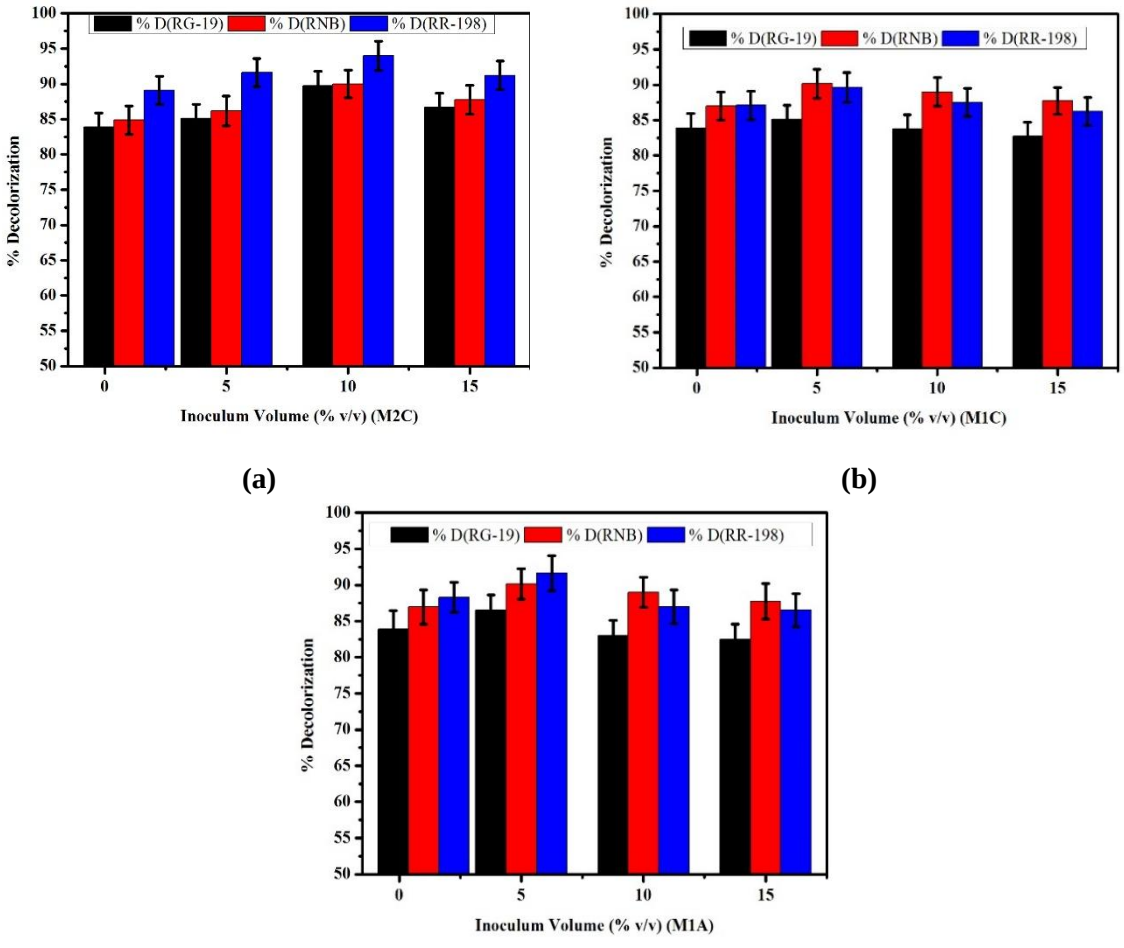


Figure 4.8: Effect of inoculum volume on dye decolorization efficiency of (a)) (M2C) *Bacillus pumilus* HKG212; (b) (M1C) *Zobellella taiwanensis* AT 1-3; (c) (M1A) *Enterococcus durans* GM13

Hence it becomes necessary to know the optimum value of inoculum volume for a batch process to enhance the overall efficiency of reaction. Figure 4.8a, 4.8b and 4.8c depicts the change in the extent of dye decolorization of all the three model dyes in response to varying inoculums size of the isolated pure cultures. M2C showed an increase in decolorization efficiency with an increase in inoculum volume from 1% (v/v) to 10% (v/v) for all the three dye (Figure 4.8a). However, further increase in inoculum volume to 15% (v/v) resulted in decreased decolorization efficiency. Similarly, for M1C & M1A (Figure 4.8b & Figure 4.8c), upto 5% (v/v) inoculum volume an increase in decolorization efficiency has been observed and further increase in inoculum volume resulted in decreased decolorization efficiency. Similar kind of results were obtained in literature for effect of varying inoculum volume on dye decolorization [214,215].

- **Effect of Aeration Condition**

Aeration condition has a tremendous impact on dye decolorization capacity of the bacterial strains. Figure 4.9a, 4.9b and 4.9c depicts the color removal efficiency of the M2C, M1C and M1A respectively under static, anaerobic and shaking conditions. Maximum decolorization was observed under anaerobic condition for M2C strain, however for M1C and M1A strain under both static and anaerobic conditions maximum decolorization was achieved which is considerably much higher than that of shaking condition. Most of the bacterial dye decolorization process of azo dyes prefers anaerobic environment as in anaerobic condition azo group of the dye acts as the electron acceptor from the reduced electron carrier i.e. NADH, quinones etc. and gets reduced [15,119,210]. Similarly under static incubation condition, oxygen transfer is limited to the broth surface only and the cell cultures sediment in the bottom of the flasks and becomes oxygen depleted [27,214] which favours degradation of azo dyes. However, if the extra-cellular environment is aerobic (shaking condition), this reduction mechanism gets inhibited by oxygen, as the oxidation of the reduced redox mediator is mediated by oxygen rather than by the azo dye [215]. Similar findings were reported by many researchers where static and anaerobic conditions favour the reductive decolorization of azo dye molecule using different bacterial species [55,216,217].

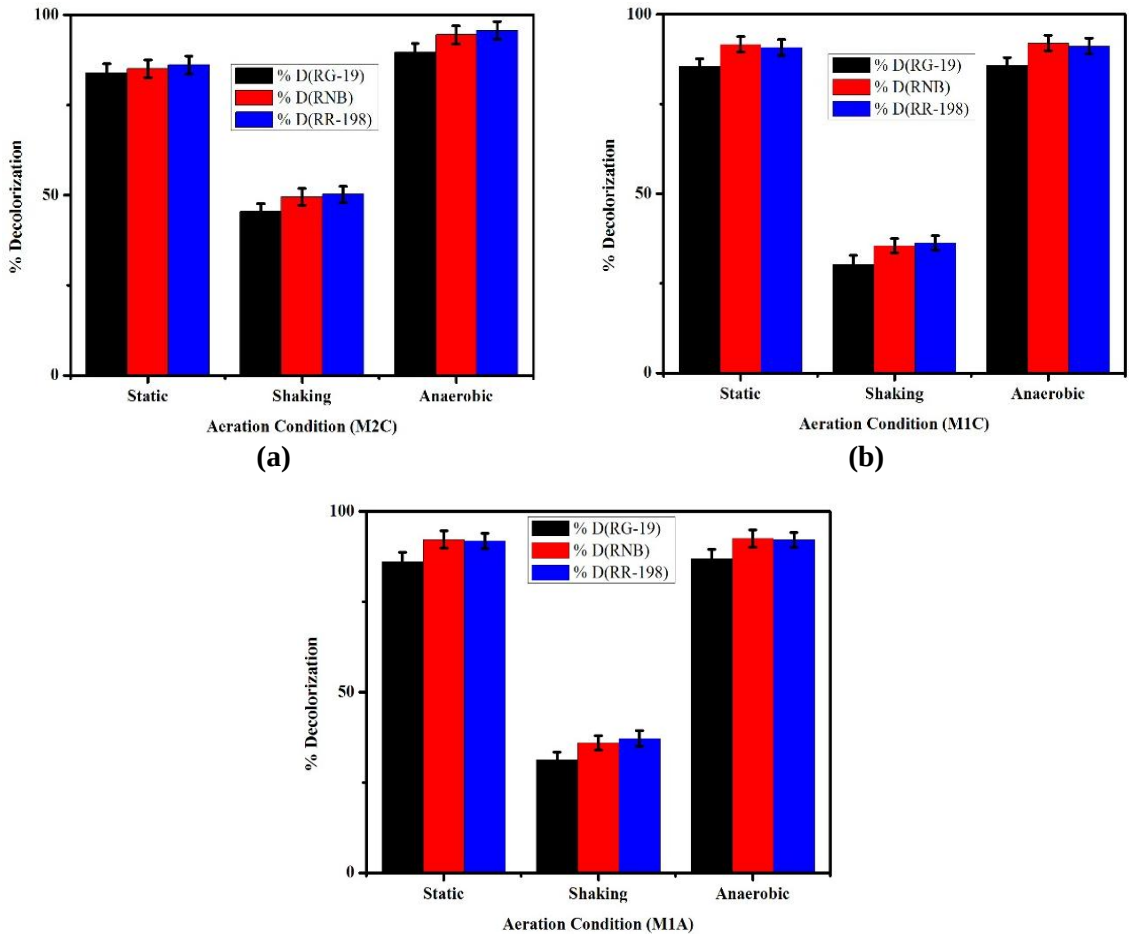


Figure 4.9: Effect of aeration condition on dye decolorization efficiency of (a) (M2C) *Bacillus pumilus* HKG212; (b) (M1C) *Zobellella taiwanensis* AT 1-3; (c) (M1A) *Enterococcus durans* GM13

- **Effect of Incubation Temperature**

Incubation temperature plays an important role in microbial growth and activity. It is one of the vital parameter taken into consideration for the optimization of any kind of bioremediation process. Batch decolorization experiments were performed at different temperatures ranging from 20 ˚C to 45 ˚C for assessing optimal decolorization capacity of isolated strains. A gradual increase in decolorization activity of our isolated bacterial strains was observed with increase in temperature from 20 ˚C to 35 ˚C. For M2C strain, maximum decolorization efficiency was obtained at 30 ˚C, whereas M1C and M1A strains showed their maximum decolorization potential at 35 ˚C. However, a sharp

decrease in color removal efficiency was observed as the temperature increased beyond 40 ˚C for all the isolates (Figure 4.10a, 4.10b and 4.10c).

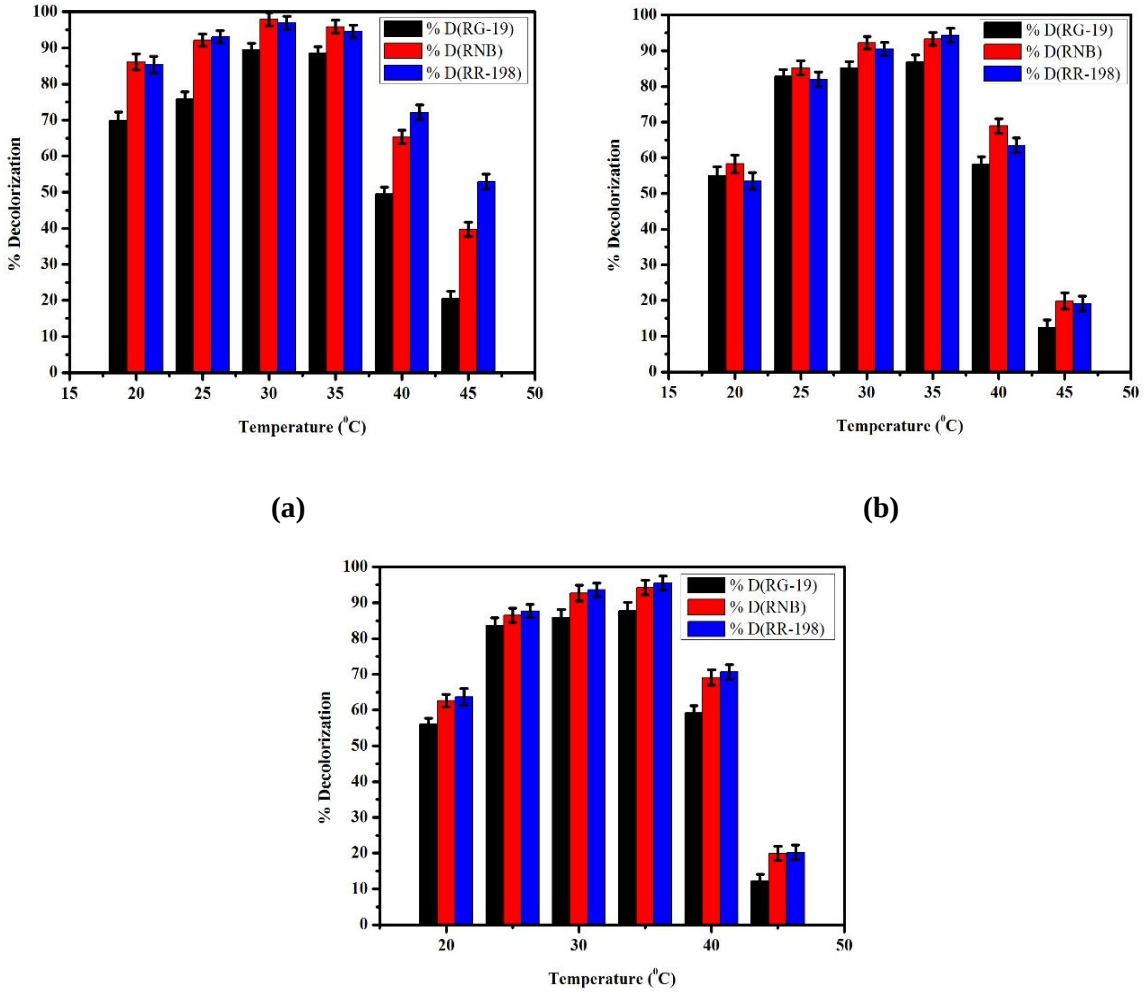


Figure 4.10: Effect of incubation temperature on dye decolorization efficiency of (a) (M2C) *Bacillus pumilus* HKG212; (b) (M1C) *Zobellella taiwanensis* AT 1-3; (c) (M1A) *Enterococcus durans* GM13

Similar to our findings, Singh et al reported that maximum decolorization of Acid Orange dye by *Staphylococcus hominis* RMLRT03 strain was observed at 35 ˚C [55]. Optimal temperature for the decolorization process using *Comamanas acidovorns*-TN1 and *Burkholdera cepace*-TN5 was reported as 35 ˚C [206]. They also reported the decrease in decolorization efficiency of the organisms with further increase in temperature.

- **Effect of Medium pH**

The hydrogen ion concentration showed profound effect on metabolic activity of microorganisms. Textile waste water shows varying pH and mostly alkaline pH is maintained during the processing as it favours the addition and substitution mechanism between the cotton fibres and azo dyes [218].

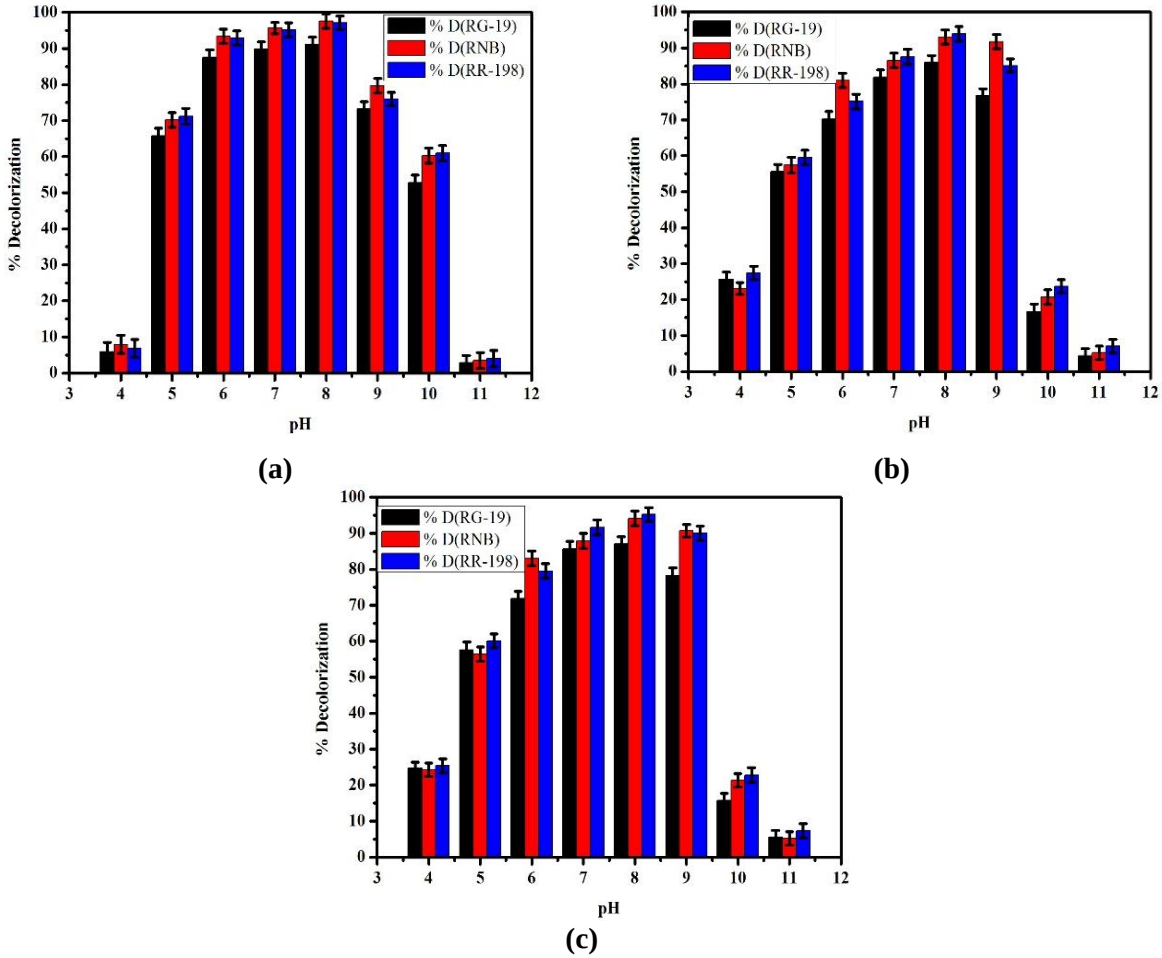


Figure 4.11: Effect of pH on dye decolorization efficiency of (a) (M2C) *Bacillus pumilus* HKG212; (b) (M1C) *Zobellella taiwanensis* AT 1-3; (c) (M1A) *Enterococcus durans* GM13

The isolated strains showed decolorization efficiency over a broad range of pH from 4-11 (Figure 4.11a, Figure 4.11b, Figure 4.11c). However, maximum decolorization was achieved at pH 8 within 24hr of incubation for all the three isolated strains. Decolourization efficiency was found to decrease significantly in higher acidic and alkaline conditions as extreme pH environment inhibits bacterial growth. Similar findings

were reported by many researchers where the optimum range of pH for textile dye decolorization using different bacterial species lies between pH 6 to pH 10 [210,219,220].

Table 4.2: Literature reports of Comparative removal of RG-19, RNB and RR-198 using different microorganisms

Culture conditions	Inoculum	Initial Concentration	% Removal	Reference
RG-19 Removal				
37 °C and pH 8, Static incubation	*Micrococcus glutamicus* NCIM-2168	50 mg/L	100% in 42hr	[125]
28 ± 2ºC and 150 rpm.	*Pleurotus pulmonarius*	100 mg/L	88.43% in 7days	[221]
30 °C, pH 3, Shaking condition	Indigenous fungal strain VITAF-1	250 mg/L	97.9% in 48hr	[222]
Shaking condition	*Emericella nidulans* TSF - 12	1000 mg/L	94.52% in 3days	[223]
30 °C, pH 8, Static incubation	*Bacillus pumilus HKG212*	100 mg/L	91.2% in 24hr	**Present Study**
RNB Removal				
Biosorption	*Pseudomonae putida*	20 mg/L to 40 mg/L	97% to 95% in 45 min	[224]
pH 6.6, 30°C, Static incubation	*T. beigelii*	50 mg/L	100% 24 hr	[225]
30 °C, pH 8, Static incubation	*Bacillus pumilus HKG212*	100 mg/L	97.59% in 24hr	**Present Study**
RR-198 Removal				
pH 7, 25°C, Static	*Alcaligenes sp.*	100 mg/L & 500 mg/L	97% & 39% in 24hr	[226]

incubation				
pH 7, 40°C, Static incubation	*Bacterial-Yeast consortium* *(Brevibacillus laterosporus and Galactomyces geotrichum)*	50 mg/L	92% in 18hr	[227]
Anoxic condition	*Aeromonas hydrophila*	100 mg/L	65% in 24hr & 100% in 7days	[210]
30◦C, 120 rpm	*A.flavus*	50 mg/L & 100 mg/L	99.11% & 96.52% in 24hr	[228]
Aerobic condition	*Brevibacterium sp.* strain VN-15	100 mg/L	99% in 120hr	[229]
30 °C, pH 8, Static incubation	*Bacillus pumilus HKG212*	100 mg/L	97.1	**Present Study**

4.1.4 Conclusion

It can be concluded from the above study that, textile waste water is a rich source of microorganisms having excellent tolerance capacity towards higher dye concentration. Three most potent bacterial strains designated as M1A, M1C and M2C were screened and isolated based on their maximum capacity to decolorize dye mixture in a wide concentration range. Through 16s rDNA molecular characterization techniques M1A strain was found to have maximum similarity with **Enterococcus durans strain GM13**, M1C strain has highest similarity with **Zobellella taiwanensis strain AT 1-3** and M2C strain has highest similarity with **Bacillus pumilus strain HKG212** based on nucleotide homology and phylogenetic analysis. All the three strains were found to be highly tolerant to higher dye concentrations and are able to achieve 80-95% decolorization in a wide range of dye concentration (i.e 100 mg/L to 1000 mg/L) for all the three model dyes (RG-19, RNB and RR-198). M2C strain (Bacillus pumilus HKG212) was found to be the best decolorizer among all the three isolates which is capable of achieving 96%, 89.5% and 94% decolorization of RNB, RG-19 and RR-198 dye respectively within 24hr of

incubation in static condition. Comparison of the dye decolorization efficiency of different microbial strains for the decolorization of RG-19, RNB and RR-198 shows that our isolated organisms has higher dye decolorization potential with efficient and faster decolorization ability for higher concentration of dyes. Effect of different physico-chemical parameters on decolorization efficiency of these three bacterial isolates was studied. All the three strains showed maximum decolorization efficiency in anaerobic and static condition. For M2C 10 % v/v inoculums volume and for M1A & M1C 5 % v/v inoculums volume was found as optimum. All the three strains showed their maximum decolorization potential in ambient temperature (30°C-35°C) and neutral to slightly alkaline pH (pH 7- pH 8) range. Considering the results obtained through this study, our further studies were carried out with these three isolated bacterial strains (M1A, M1C, M2C).

4.2 Optimization of dye decolorization efficiency of developed consortia through response surface methodology and kinetic studies

4.2.1 Decolorization Study Using Individual and Consortium of Organisms

The ability of pure cultures as well as the developed consortium to decolorize RG-19 dye was studied and the results are shown in Figure 4.12. Both the pure cultures as well as the microbial consortium were found to be very effective in decolorizing RG-19 dye in a wide concentration range (100 mg/L – 1000 mg/L). However, the two developed consortia were found to be more efficient in comparison to the individual pure cultures. At lower dye concentration (100 mg/L) maximum of 86.80% ,89.50% and 87.20% decolorization was noticed after 24hr incubation, which further get reduced to 81%, 85% and 83.19% at higher concentration (1000 mg/L) for pure cultures M1C, M2C and M1A respectively. While at 93% and 95.52% decolorization was achieved with “M12C” and “MA12C” consortium respectively at the same condition within 24 h at 30 °C at 100 mg/L RG-19 concentration. It was noticed that with higher concentration of dye (i.e. 800 mg/L-1000 mg/L), around 86-90 % decolorization of RG-19 has been achieved with both

the consortia which is higher than that achieved with pure cultures. Several reports are available which supports our results, where consortiums of microbes were found to be more effective in decolorization of various textile dyes as compared to the individual pure cultures [25,132,135,201]. Saratale et al. (2010) reported higher decolorization activity of reactive azo dye Green HE4BD by developed consortium GR of *P. vulgaris* and *M.glutamicus* as compared to its constituent pure strains. Phugare et al. (2011) reported faster decolorization and degradation of Red HE3B dye with a bacterial consortium comprising of *Providencia sp.* SDS and *P. aeuroginosa* compared to the individual pure strains. Besides, Lade et al. (2012) obtained enhanced decolorization of azo dye Rubine GFL using a fungal-bacterial consortium-AP of *A. ochraceus* and *Pseudomonas sp.* as compared to the individual microorganisms. This enhanced decolorization results shown by the consortium might be attributed to the synergistic reactions of individual strains in the consortium [230]. Moreover, the pure strains in microbial consortium may attack the dye molecules at various position or may utilize the degradation metabolites generated by co-existing strains for further degradation that results in enhanced decolorization of dyes [231,232].

Hence, considering these facts, further studies were carried out with these two microbial consortia "M12C" and "MA12C". Different process parameters were optimized to achieve maximum decolorization efficiency of the developed consortia.

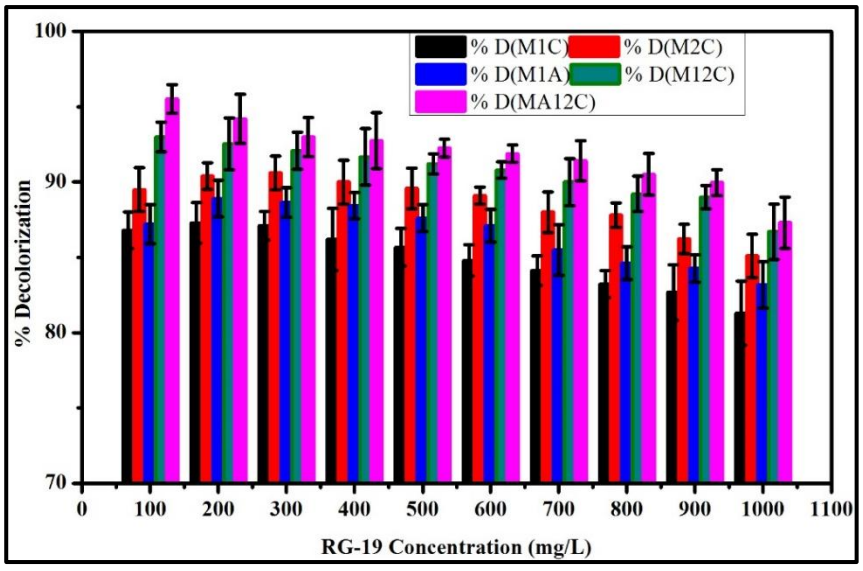


Figure 4.12: Comparative decolorization capacity of Individual pure cultures and developed consortia (at 30°C and pH 7.5) for decolorization of RG-19

4.2.2 Optimization of Culture Condition of "M12C" Consortium to Achieve Improved Decolorization of RG-19

The first part of the investigation includes the study of decolorization capacity of "M12C" consortium and optimization of the process parameters affecting its dye decolorization efficiency. An industrially important azo dye Reactive green-19 (RG-19) has been used as the model dye for this study. Though, very few reports are available on decolorization of RG-19 dye using different microorganisms (i.e. algae, fungi, and bacteria) most of them incorporate pure cultures [125,210,223,233,234]. However, to best of our knowledge, this could be the first report on decolorization study of RG-19 using a developed bacterial consortium comprising of organisms isolated from dye contaminated wastewater. RSM based Box-Behnken design was employed for process optimization in terms of decolorization efficiency. Kinetic parameters for the decolorization of RG-19 were estimated at the optimum conditions using the developed consortium. Degradation of RG-19 was confirmed by evaluating the metabolites formed after decolorization using UV-Vis spectrophotometer, FTIR, HPLC and GC-MS analysis.

- **Effect of Individual Process Parameters**

In bioremediation process, physico-chemical parameters like pH of culture media, incubation temperature, inoculum volume and composition of culture media are considered as the vital components that affect the overall efficiency of the process [210,213]. Study of individual effect of each parameter is essential before analyzing the synergistic effect of these factors.

- **Effect of inoculums volume (%v/v)**

Culture media containing 100 mg/L concentration of RG-19 dye has been inoculated with different inoculums volume (0.1 %v/v to 3 %v/v) of M12C consortium. Figure 4.13 clearly shows that, there is no apparent effect of inoculums volume on decolorization efficiency of the consortium. Almost 93% decolorization of RG-19 has been achieved with all tested inoculum volumes.

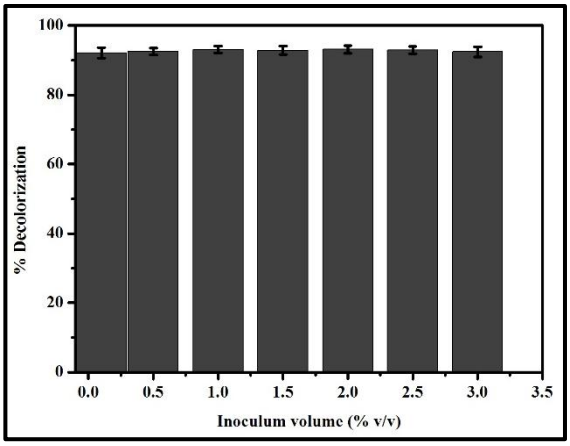


Figure 4.13: Effect of inoculums volume (%v/v) on dye decolorization efficiency of "M12C" (at 100 mg/L; 30 °C)

- **Effect of pH**

Decolorization capacity of the developed consortium was analysed at wide range of pH (5-10). The developed consortium effectively decolorized (above 55%) RG-19 dye in the pH range of 5-10 and maximum decolorization of 94.10% was observed at pH 8 (Figure 4.14). Similar results have been reported by many researchers where maximum decolorization of various textile dyes were achieved at neutral-to-slightly alkaline pH using different bacterial species [23,235]. Phugare et al.(2011) observed that, pH 7 was optimum for decolorization of azo dye RED HE3B by consortium of *Providencia sp.* SDS and *P. aeuroginosa* strain [25]. The overall biochemical process is directly associated with pH and more likely related to transport of dye molecules and reducing equivalents across the cell membrane, which is the rate limiting factor in decolorization study.

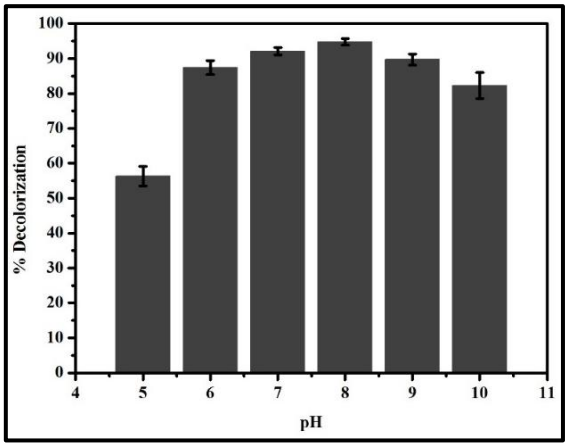


Figure 4.14: Effect of pH on dye decolorization efficiency of "M12C" (at 100 mg/L; 30 °C)

- **Effect of Temperature**

Incubation temperature was found to have profound effect on the efficacy of the consortium to decolorize RG-19 dye. Significant decolorization in a broad range (around 30% to 93%) was obtained in the temperature range of 25 °C-40 °C and the uppermost value for decolorization (i.e. 93.08%) was found at 30 °C after 24 h of incubation (Figure 4.15). At temperature below 25 °C and above 40 °C, a considerable decrease in decolorization was noticed suggesting the acute effect of temperature on dye removal by the consortium.

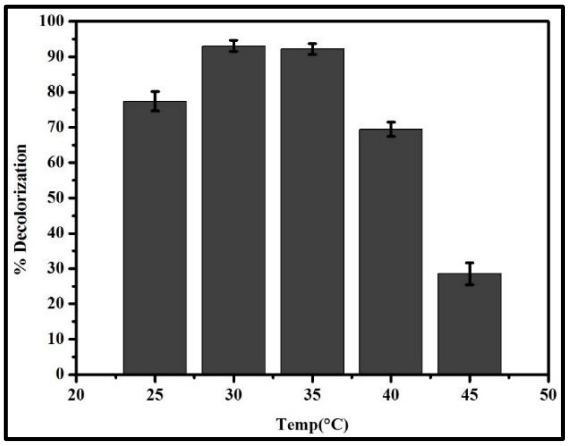


Figure 4.15: Effect of Temperature on dye decolorization efficiency of "M12C" (at 100 mg/L; pH 7.2)

These results suggest that the bacterial consortium is mesophilic in nature and similar results have been reported earlier where mesophilic bacteria are known to degrade azo dye [23,235]. Moreover, this result agrees to an earlier report on decolorization of Fast Red by Bacillus subtilis where maximum degradation occurred at 30-35°C. Temperature above 30°C lead to decline in decolorization ability due to loss of cell viability or deactivation of organism involved in decolorization process.

- **Effect of Co-Substrates**

Microorganisms are known to utilize a variety of complex compounds as their sole carbon source but only few reports have been made on successful utilization of dye [194]. In the present study the bacterial consortium was not able to utilize RG-19 dye as a sole source of carbon and energy source. Hence we investigated different combination of carbon and

nitrogen sources as co-substrate supporting dye decolorization efficiency of our developed consortium M12C.

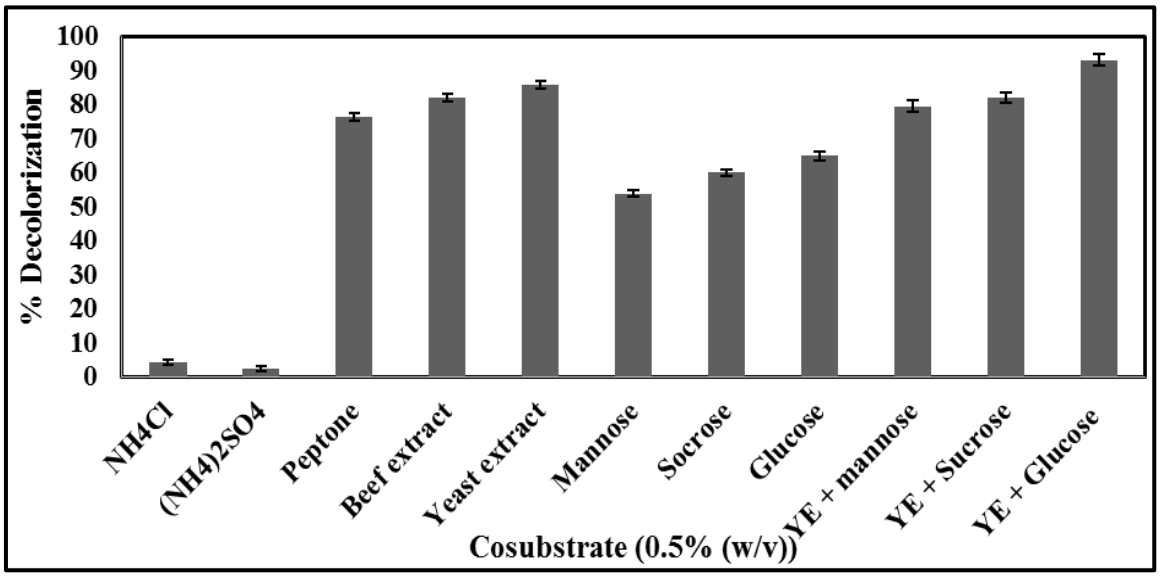


Figure 4.16: Effect of different co-substrates on decolorization efficiency of M12C consortium (at 100 mg/L; pH 7.2)

Among the different carbon and nitrogen sources, Glucose and Yeast extract were found to be the best carbon and nitrogen source respectively enhancing the efficiency of decolorization (Figure 4.16) of M12C consortium. With the presence of only Yeast extract in the medium, 85.87% decolorization has been achieved which increased to 93.13% with addition of Glucose as the additional carbon source. Hence, presence of both Glucose and Yeast extract in the medium were found to be essential for achieving enhanced decolorization of RG-19 using M12C. Glucose has been reported as the best carbon source for supporting maximum dye decolorization by many researchers [55,200]. Yeast extract has been the most commonly used nitrogen source for most of the decolorization processes using bacteria [55,236].

Amount of both the co-substrates, glucose and yeast extract in the minimal salt media (at 30 °C and pH 7.2) were analysed for their ability to enhance dye decolorization in the range of 0-2.5 (g/100mL) and the effect on percentage decolorization has been shown (see Figure 4.17 and Figure 4.18).

- **Effect of glucose concentration**

According to results (Figure 4.17), on increasing the amount of glucose from 0-1.5 (g/100mL), there was a corresponding increase in % decolorization but any further increase in carbon source beyond 1.5 g/100mL was found to have a no apparent effect on decolorization efficiency of the consortium. In the absence of yeast extract, with glucose as a co-substrate, the percentage decolorization decreased to 65%, where as in the absence of glucose in the medium, up to 85% decolorization could still be achieved with only yeast extract as the co-substrate (Figure 4.16). Hence, the glucose amount was fixed at 1.5 (g/100mL) throughout the study and varying amount of yeast extract was analysed for its effect on decolorization.

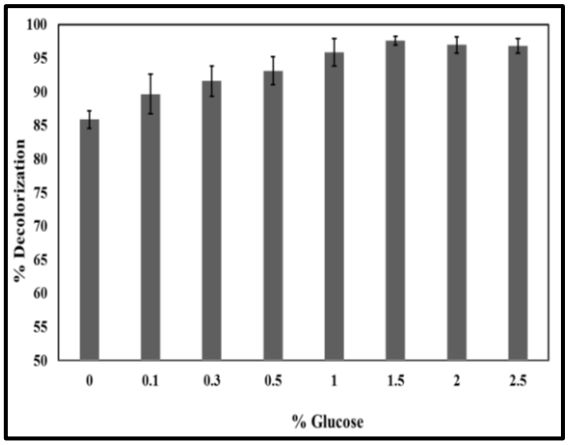


Figure 4.17: Effect of Glucose on dye decolorization efficiency of "M12C" (at 100 mg/L dye; pH 7.2; 30 °C)

- **Effect of yeast extract concentration**

In comparison to glucose, yeast extract was found to have a tremendous effect on decolorization of RG-19 dye by the consortium. This is quite evident from the curve found in the yeast extract utilization graph (Figure 4.18) rather than almost a straight line found in the case of glucose utilization graph (Figure 4.17). The metabolism of organic nitrogen sources like yeast extract is essential for the regeneration of NADH that acts as an electron donor for the reduction of azo bonds [32]. As we increased the amount of yeast extract in the medium from 0-1 (g/100mL), the percentage decolorization of RG-19 dye increased significantly (i.e. from 65%-97.11%). However, further increase in yeast

extract concentration beyond 1 g/100mL appears to have negative effect on decolorization.

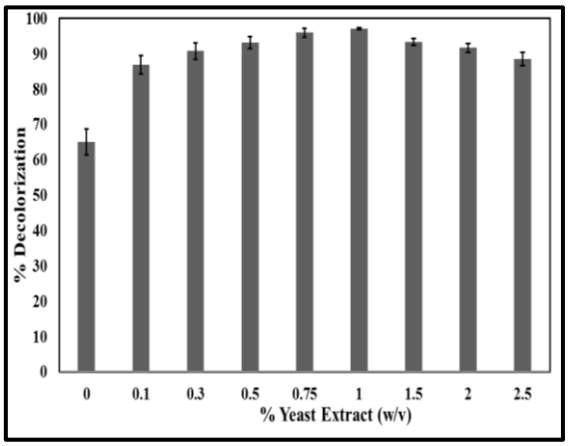


Figure 4.18: Effect of Yeast Extract on dye decolorization efficiency of "M12C" (at 100 mg/L dye; pH 7.2; 30 °C)

Hence, for optimization study of physico-chemical parameters a temperature ranges of 25 °C-45 °C, pH 5-10, and yeast extract concentration 0-1.5 (g/100mL) were selected to carry out a systematic study of the dye decolorization process through response surface methodology and Box-Bhenken design.

- **Process Optimization Using Response Surface Methodology (RSM)**
  - **Box-Behnken analysis**

Based on the results obtained from preliminary analysis of bio-decolorization process variables and supporting literatures [23,130,135,236–238], three independent process parameters incubation temperature, pH, and concentration of yeast extract were chosen as the important parameters which affect the decolorization process. In order to study the synergic effect of these factors, experiments were performed for different combinations of the physical parameters using statistically designed experiments. The 17 experimental runs (consisting of 12 trials plus 5-centre points) were carried out to obtain a quadratic model for decolorization of RG-19 dye with a developed microbial consortium according to the Box-Behnken design. Each run was performed in triplicate to verify the reproducibility and the mean values for % decolorization of RG-19 dye are presented in Table 4.3, where the predicted values of response were obtained from quadratic model fitting techniques. The proposed second degree polynomial was fitted to the data obtained

using multiple linear regressions to determine the optimum physiological conditions that resulted in the maximum decolorization of RG-19 dye. The effects of broth pH, incubation temperature and yeast extract concentration were quantitatively evaluated using response surface curves.

The predicted response, *Y* can be obtained and given as:

$$\boldsymbol{Y = +93.54 - 17.94A + 5.96B + 11.60C - 3.40AB - 6.24AC + 6.56BC - 35.95A^2 - 18.23B^2 - 19.02C^2} \quad (4.1)$$

Where Y is the predicted response and A, B, C are the coded values of the independent variables incubation temperature (°C), pH and concentration of yeast extract (g/100 mL) respectively.

Table 4.3: Three factor Box-Behnken design with experimental as well as predicted responses of dependent variable for RG-19 decolorization using microbial consortium "M12C"

Runs	Factors						Response		Internally Studentized Residual
	Coded values			Actual values			% of decolorization		
	A	B	C	A	B	C	Observed	Predicted	
1	0	0	0	35	7.5	0.75	92.10	93.54	-0.724
2	+1	0	+1	45	7.5	1.5	24.45	25.98	-1.379
3	-1	+1	0	25	10	0.75	64.12	66.66	-2.289
4	0	+1	-1	35	10	0	45.10	44.09	0.910
5	0	0	0	35	7.5	0.75	95.00	93.54	0.734
6	0	-1	+1	35	5	1.5	54.36	55.37	-0.910
7	+1	-1	0	45	5	0.75	21.40	18.86	2.289
8	0	-1	-1	35	5	0	43.70	45.29	-1.430
9	0	+1	+1	35	10	1.5	82.00	80.41	1.430
10	+1	+1	0	45	10	0.75	23.92	23.98	-0.051
11	0	0	0	35	7.5	0.75	92.50	93.54	-0.523

12	-1	0	-1	25	7.5	0	40.20	38.67	1.379
13	-1	0	+1	25	7.5	1.5	75.30	74.34	0.859
14	-1	-1	0	25	5	0.75	48.00	47.94	0.051
15	+1	0	-1	45	7.5	0	14.30	15.25	-0.859
16	0	0	0	35	7.5	0.75	93.00	93.54	-0.272
17	0	0	0	35	7.5	0.75	95.10	93.54	0.785

- **Model Adequacy**

The sparsity-of-effects principle states that a multivariable system is usually dominated by main effects and low-order interactions (higher-order interactions such as three-factor interactions are very rare) [239,240]. Hence, we have considered two-way interactions for the involved system. Linear, interactive, quadratic and cubic models were fitted to the experimental data to obtain the regression equations. Sequential model Sum of Squares, Lack of Fit Test and Model Summary Statistics were carried out to decide about the competency of the model among various models to represent RG-19 dye removal using the consortium, and the results are given in Table 4.4. All p-values for lack-of-fit (larger than 0.05 for all responses) indicate that there is insufficient evidence to suggest that the model does not adequately fit the data. In other words, the form of the model chosen to explain the relationship between the factors and the response is correct [239–241]. Cubic model was found to be aliased. Quadratic model showed that the p value was lower than 0.01 for the Sequential model sum of squares (Table 4.4) and had an insignificant lack of fit among others. Model summary statistics as well showed that the excluding cubic model was aliased while the quadratic model was found to have the maximum "Adjusted R-Squared" and the "Predicted R-Squared" values. Later, ANOVA shows that all three linear terms (A, B, C) and three 2FI term (AB, AC, BC) are significant at the 0.05 probability level and hence were included in the final model to maintain model hierarchy [239].

Thus, the quadratic model includes lower-order linear and two-factor interaction terms, which lay down the foundation to make use of this entire model for predicting the response. Also the quadratic model produces the least PRESS (predicted residual sum of

squares) which shows a good measure of its relative precision for forecasting future outcomes [239]. Therefore, provisionally quadratic model was chosen for further analysis.

Table 4.4: Adequacy of the model for RG-19 decolorization using "M12C"

Source	Sum of Squares	*df*	Mean Square	F Value	p-value Prob > F	Remarks
			Sequential model sum of squares			
Mean versus Total	59360.04	1	59360.04			
Linear versus Mean	3936.71	3	1312.24	1.77	0.2016	
2FI versus Linear	374.00	3	124.67	0.13	0.9370	
Quadratic versus 2FI	9207.98	3	3069.33	621.42	<0.0001	Suggested
Cubic versus Quadratic	26.56	3	8.85	4.42	0.0925	Aliased
Residual	8.01	4	2.00			
Total	72913.30	17	4289.02			
Source	**Sum of Squares**	***df***	**Mean Square**	**F Value**	**p-value Prob > F**	**Remarks**
			Lack of Fit Tests			
Linear	9608.54	9	1067.62	533.01	<0.0001	
2FI	9234.54	6	1539.09	768.39	<0.0001	
Quadratic	26.56	3	8.85	4.42	0.0925	Suggested
Cubic	0	0				Aliased
Pure Error	8.01	4	2.00			
Source	**Std. Dev.**	**R-Squared**	**Adjusted R-Squared**	**Predicted R-Squared**	**PRESS**	**Remarks**
			Model Summary statistics			
Linear	27.20	0.2905	0.1267	-0.0625	14400.10	
2FI	30.40	0.3181	-0.0911	-0.7462	23666.99	
Quadratic	2.22	0.9974	0.9942	0.9677	437.52	Suggested
Cubic	1.42	0.9994	0.9976		+	Aliased

+ Case(s) with leverage of 1.0000: PRESS statistic not defined

- **Fitting of Second-Order Polynomial Equation and Statistical Analysis**

The significance of the fit of the second-order polynomial for the percentage of decolorization of RG-19 dye was assessed by carrying out the analysis of variance (ANOVA) and the results are shown in Table 4.5. A p-value lower than 0.05 indicates that the model is significant and p-value higher than 0.1000 indicates that the model is not significant [239,240,242]. The model F-value of 304.11 and the corresponding p-value of <0.0001implies the model is significant and there is only 0.01% chance that the F-value is large which could occur due to noise. The lack-of-fit term in the residual indicates the variation due to model inadequacy. The lack of fit F-value of 4.42 is not significant as the corresponding p-value is 0.0925 (>0.05), to fit the model we need an insignificant lack of fit. The P-value of linear terms A, B and C ($p << 0.05$) also suggests that there is significant linear effects of temperature, pH and yeast extract concentration on decolorization. Also the three 2FI term (AB, BC and AC) turns out to be significant at the 0.05 probability level. This indicates that the interaction effect of each independent variable with each other are significant and influences the efficiency of decolorization of RG-19 dye. The model also contains three squared effects (A^2, B^2, and C^2). Squared terms are used to evaluate whether or not there is curvature in the response surface. The P-Value of quadratic effects of A, B and C are less than 0.05 and hence render significant quadratic effects. That is the relationship of incubation temperature, broth pH and yeast extract concentration with % decolorization of RG-19 dye followed a curved line rather than a straight line. A model small p-value (Prob > F) of terms indicates that selected model terms can improve the model significance. The ANOVA statistical analysis revealed that all the independent process variables had significant effect on the decolorization of RG-19 dye by the developed consortium.

Table 4.5: Summary of analysis of variance results; ANOVA for response surface quadratic model of % of Decolorization of RG-19 using microbial consortium "M12C"

Factors	**Sum of Squares**	**Degree of freedom**	**Mean Square**	***F*-value**	***P*-value Prob > F**	**Remarks**
Model	13518.69	9	1502.08	304.11	< 0.0001	significant
A-Temperature	2575.83	1	2575.83	521.51	< 0.0001	

B-pH	284.17	1	284.17	57.53	0.0001	
C-Yeast Extract	1076.71	1	1076.71	217.99	< 0.0001	
AB	46.24	1	46.24	9.36	0.0183	
AC	155.63	1	155.63	31.51	0.0008	
BC	172.13	1	172.13	34.85	0.0006	
A^2	5442.83	1	5442.83	1101.97	< 0.0001	
B^2	1398.72	1	1398.72	283.19	< 0.0001	
C^2	1523.80	1	1523.80	308.51	< 0.0001	
Residual	34.57	7	4.94			
Lack of Fit	26.56	3	8.85	4.42	0.0925	not significant
Pure Error	8.01	4	2.00			
Correlation Total	13553.26	16				

Table 4.6: Statistical summary of quadratic model for decolorization of RG-19 decolorization using "M12C"

Standard Deviation	2.22	R-Squared	0.9974
Mean	59.09	Adj. R-Squared	0.9942
C.V. %	3.76	Pred. R-Squared	0.9677
PRESS	437.52	Adeq. Precision	45.928

The coefficient of determination R^2 is defined as the ratio of the explained variation to the total variation and represents a measure of the degree of fit. A good model fit is expected to yield an R-squared value of at least 0.8 [243]. Hence, an R-squared value of 0.9974 indicates a high degree of correlation between the observed and predicted responses. The predicted R^2 of 0.9677 is in reasonable agreement with the Adj.R^2 of 0.9942. A close value of R-squared and Adj.R^2 indicates the adequacy of the model. Adequate precision measures the signal to noise ratio and the ratio greater than 4 is considered desirable

[239]. The ratio of 45.928 indicates an adequate signal. Hence, quadratic model can be used to navigate the design space. The coefficient of variation (C.V) is the ratio of the standard error of estimate to the mean value of the observed response. C.V < 10% indicates the model to be reasonably reliable and reproducible [240]. Hence C.V. % corresponding to 3.76 together with low standard deviation of 2.22 indicates a high level of accuracy and an excellent consistency of the model for the experimental results (Table 4.6).

Figure 4.19a shows the Box-Cox plot of power transforms for % decolorization of RG-19 dye using the bacterial consortium. Logarithm of residuals of sum of square against λ should dip fairly steeply with a minimum in the region of optimum value [244]. The value of current λ should lie in between the low C.I (confidence interval) and high C.I values [240]. The minimum confidence and maximum confidence interval for the model are 0.23 and 1.35 respectively and the value of current point of C.I. (λ = 1) falls close to the best lambda value of 0.71 and is within the C.I. which indicates no transformation of model is required.

The perturbation plot (Figure 4.19b) explains the effect of each factor on the process of decolorization graphically. A steep slope or curvature in a factor shows that the response is sensitive to that factor. A relatively flat line shows insensitivity to change in that particular factor [239,241]. It can be concluded from the Figure 4.19 that each variable used in the present study is having its individual effect on the process. Gradual increase in temperature, pH and yeast extract concentration from low (-1) to high level (+1) resulted in corresponding increase and decrease of % Decolorization of RG-19 dye.

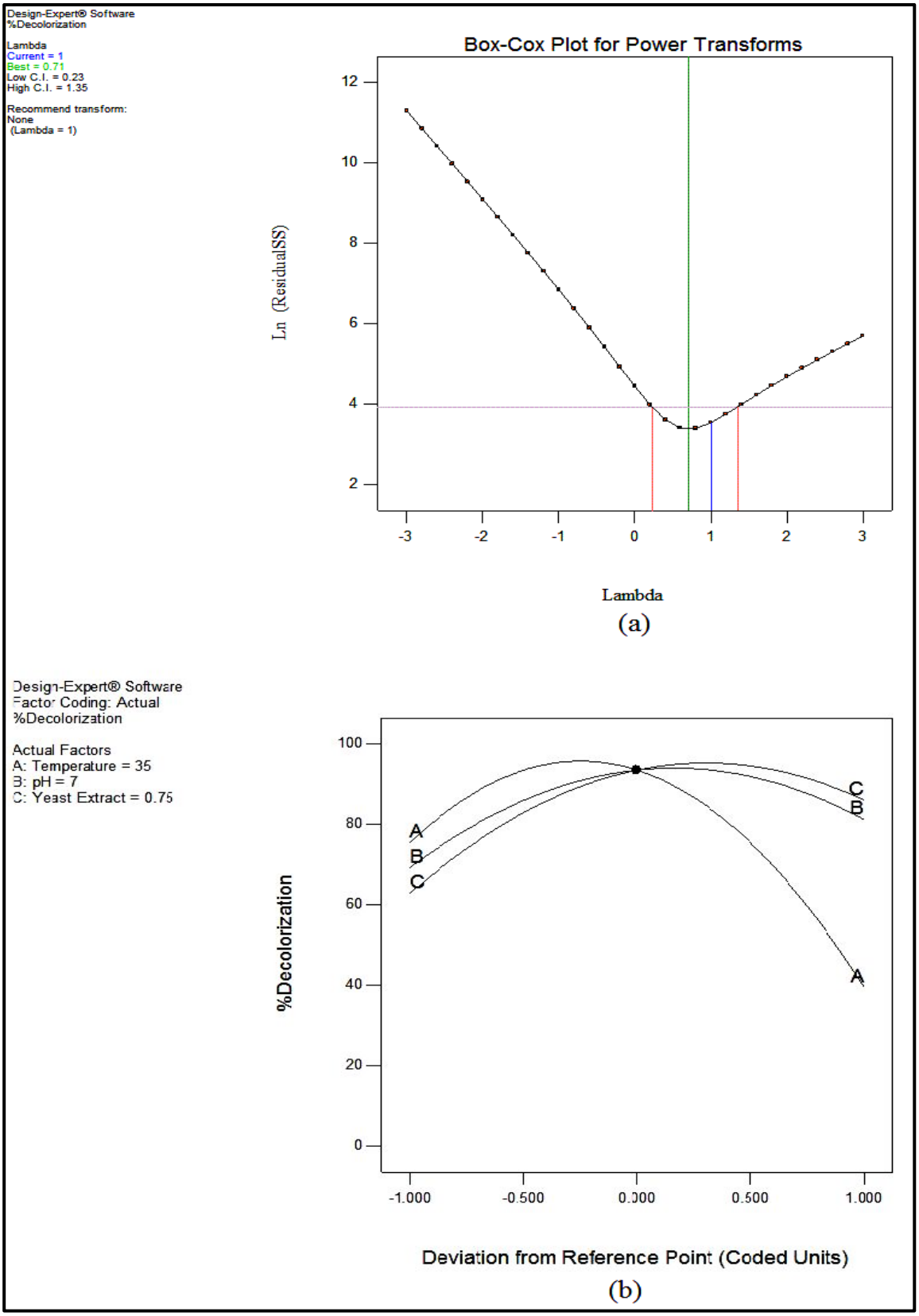


Figure 4.19: (a) Box-Cox Plot of Power Transforms (b) Perturbation graph for percentage of decolorization of RG-19 using microbial consortium "M12C"

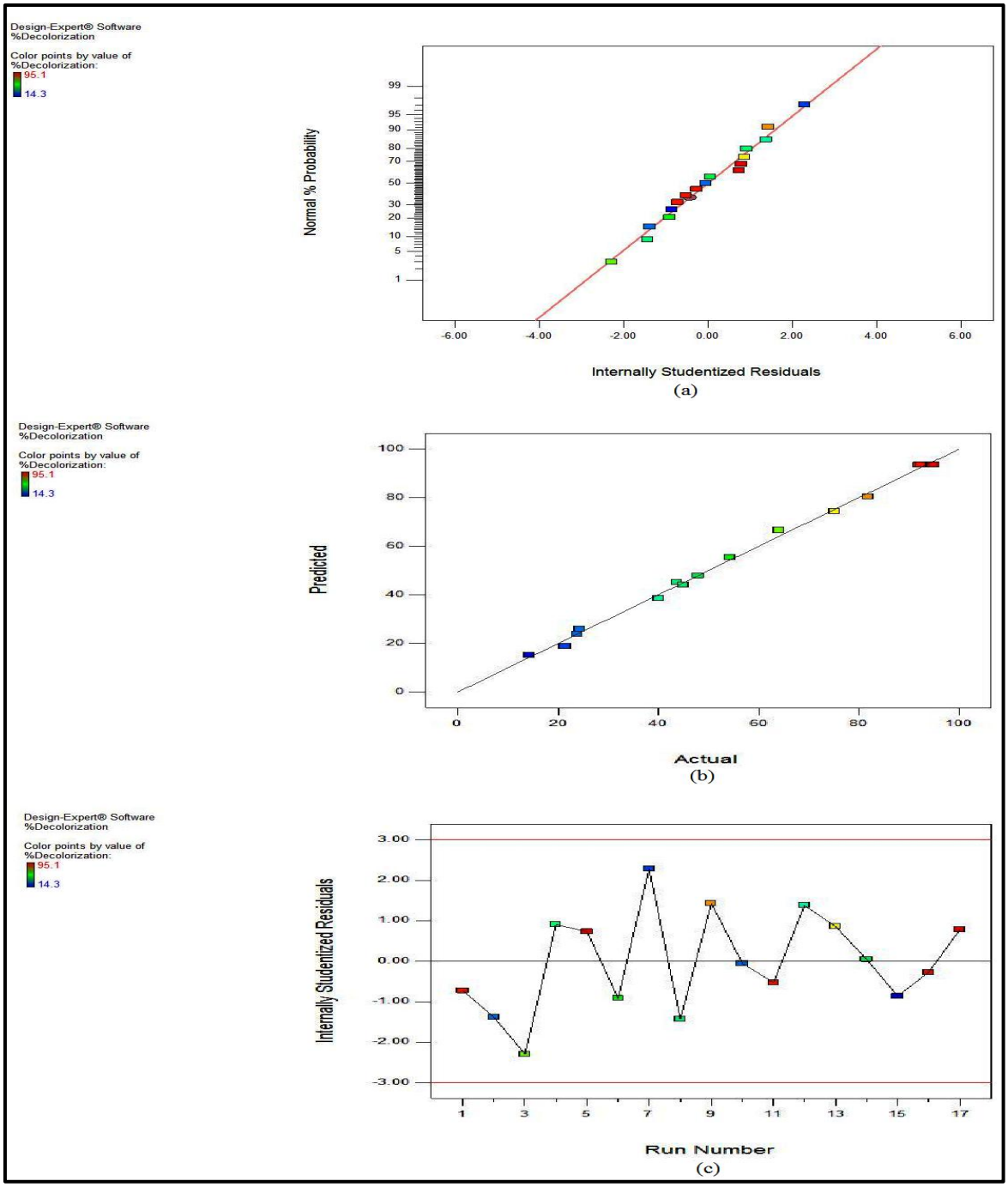


Figure 4.20: (a) Normal plot of residuals (b) Plot showing the correlation between the predicted and actual experimental values (c) plot of the residuals versus the experimental run order of model for decolorization of RG-19 dye using microbial consortium "M12C"

Figure 4.20a shows normal probability versus internally studentized residuals for the used model. To judge the model adequacy residuals from the least square fit plays an important role. The normal probability plot of the residuals is used to detect and explain, the

assumptions. It states that, errors are normally distributed and are independent of each other, and that the error variance is homogeneous and true [245]. The residual plot is approximately a straight line which indicates that there is no serious violation of assumptions underlying the analysis and that a transformation of the response is not required [240].

Figure 4.20b shows the linear relationship between the predicted and actual experimental values of % decolorization of RG-19. Actual values were determined from particular runs, and predicted values were calculated from the approximating function used for the model. Less deviation between the experimental and predicted values and the point clusters around the diagonal line indicates a good fit of the model [246].

The Residual vs. Run plot can be used to check whether the response is affected by the sequence of the test runs. The validity of our model depends on the error being randomly dispersed. Because there is no obvious pattern in Figure 4.20c, we can conclude that the results are not affected by the test sequence. The two dashed red lines are the critical values (+3 to -3) at a significance level of 0.1. Their values are calculated based on the SEV (smallest extreme value) distribution. If a residual point is beyond these two lines, it means the model cannot fit that observation very well [239]. In Figure 4.20c, we can see there is no point far below the lower critical value.

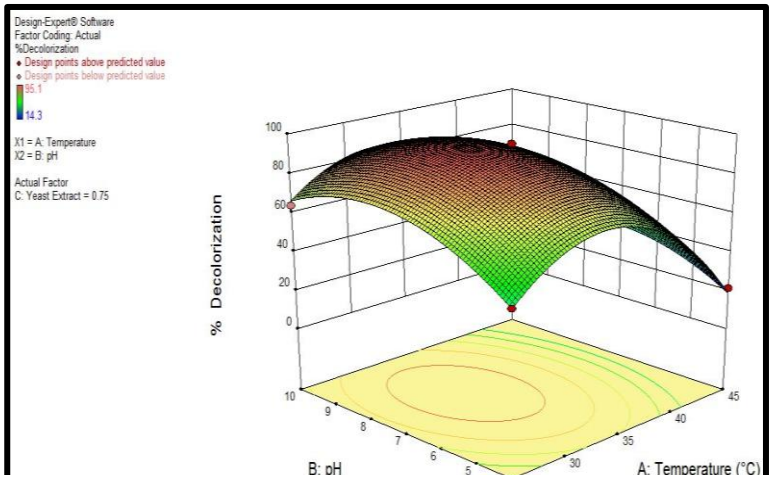
- **Effect of Various Parameters on RG-19 Dye Removal Efficiency**

The three-dimensional response plots are the graphical representations of the regression equation. Optimum values for each variable can be tracked from these plots to maximize the response. The relationship between the % Decolorization of RG-19 dye and the three independent factors (incubation temperature, pH and yeast extract concentration) are shown through 3D surface plots and the corresponding contour plots (Figure 4.21a, Figure 4.21b, and Figure 4.21c). Each plot shows the effect of two variables within their studied ranges, with the other variable fixed to the zero level. The nature and extents of interactions between two independent variables are predicted from the shape of the contour plots. An elliptical contour plot indicates a prominent interaction, where as a negligible effect appears as a circular contour plot [240].

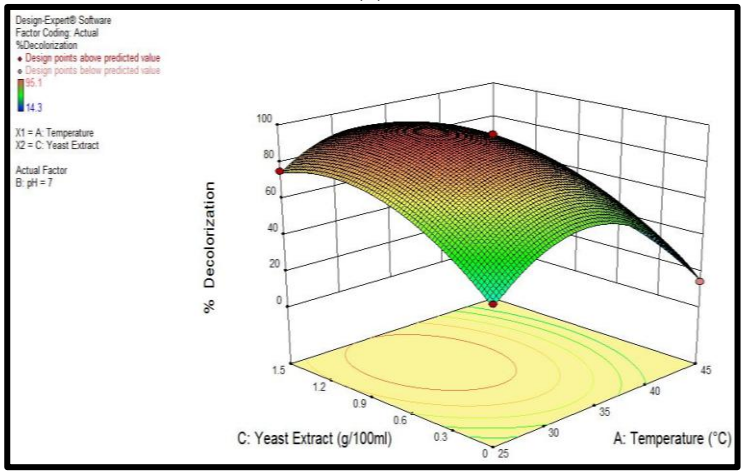
Figure 4.21a shows the three-dimensional surface plot and the corresponding contour plot for the effect of temperature and pH on percentage of decolorization of RG-19 dye. It is evident from the figures that the decolorization increases with increase in temperature and from around 30 °C to 35 °C with maximum percentage of decolorization achieved though further increase in temperature that lead to decrease in decolorization efficacy. This indicates that the organisms consisting of the consortium are mesophiles and hence grow efficiently at ambient temperature and at higher temperature cells either dies or loses their activity to decolorize the dye. Similar finding have been reported where bacterial isolates were utilized to decolorize textile azo dyes and mesophilic temperature range was found to be very effective for obtaining maximum color removal efficiency [23,247,248]. The linear term effect of pH also shows that maximum decolorization was obtained in the pH range of around 6.5 to 8.5 and further increase or decrease of pH beyond this range leads to decrease in percentage of decolorization. Since bacterial strains are known to be sensitive to the initial pH and temperature of the medium. A substantial variation in pH and temperature values may influence the three-dimensional shape of the enzymes in the microorganisms and hence its usability [23]. The elliptical shape of the contour plot suggests that there is a significant quadratic effect of temperature and pH but only in the mid-range of parameters for decolorization of RG-19 dye.

Three-dimensional surface plot in Figure 4.21b displays the interactive effect of temperature with Yeast extract (YE) on percentage decolorization of RG-19 dye. This plot shows that as we move from low to mid temperature zone with a gradual increase in YE concentration, the decolorization efficacy of consortium increases for RG-19 dye.

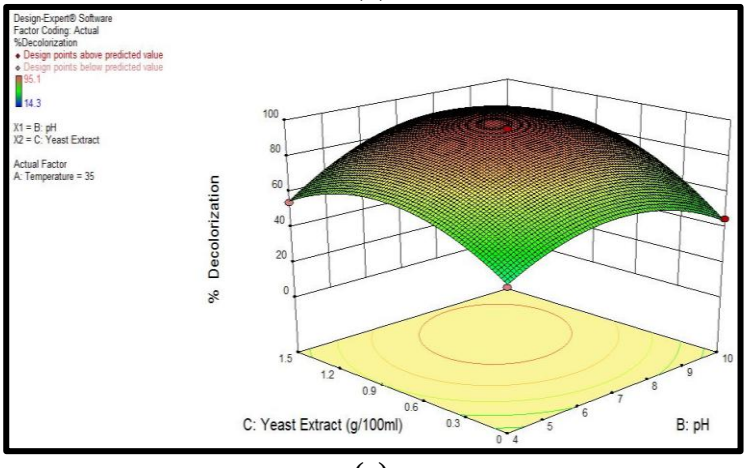
Figure 4.21c represent the surface plot of response for the synergistic effect of pH and YE concentration. The plot shows that a gradual increase in the YE concentration has positive incremental effect on decolorization capacity of the consortium for RG-19 dye. The consortium seems to utilize YE as a co-substrate quite effectively for regeneration of NADH with significant results beyond 0.6 g/100ml. Yeast extract has been reported for its importance in azo-dye decolorization, and the current results agree with the earlier findings [13,55,236]. The curve of the surface plot shows the impact of synergic effect of pH and Yeast Extract as we move towards higher concentration of YE (above 0.6 g/100mL) and pH.



(a)



(b)



(c)

Figure 4.21: Three-dimensional response surface plot for the effect of (a) pH and Temperature (b) Yeast Extract and Temperature (c) Yeast Extract and pH on percentage of RG-19 dye decolorization using microbial consortium "M12C"

The graphical 3-D view of the decolorization process shows very clearly the synergic effect of process parameters and also indicates that the experimental value must consider the running effect of these significant factors at the stipulated levels to maximize dye decolorization by the consortium.

- **Model Validation and Confirmation**

To validate the optimized combination of the variables obtained through RSM, few confirmatory experiments were carried out (see Table 4.7). Verification experiments were performed at the predicted conditions to ascertain validity and adequacy of the models. One of the solutions was then chosen for further process studies to confirm the validity of the statistical experimental strategies with experimental data. The optimal decolorization conditions were as follows: incubation temperature, 32.04 °C; pH, 8.3 and Yeast Extract concentration, 1.16 mg/100mL. The average decolorization rate achieved at this condition was 97.10 % within 24 h of incubation. The observed confirmatory experiment results were found to be very close and in good agreement with the predicted results. As a result, the model developed was considered to be accurate and reliable.

Table 4.7: Results of validation experiments conducted for model validation and confirmation at optimum conditions as obtained from RSM

Solution	Temperature	pH	Yeast Extract	% Decolorization		Desirability
				Predicted	Actual	
1	29.57	8.54	1.19	96.61	94.10	1.00
2	30.82	8.56	1.02	98.26	95.70	1.00
3	32.78	8.83	0.80	95.16	92.20	1.00
4	33.67	8.06	0.92	97.97	95.10	1.00
5	**32.04**	**8.35**	**1.16**	**98.88**	**97.10**	**1.00 Selected**
6	29.16	8.16	0.97	95.90	92.22	1.00
7	31.00	9.00	1.20	96.77	93.70	1.00
8	31.30	8.78	0.80	95.32	92.00	1.00
9	33.32	7.20	0.98	96.34	93.10	1.00
10	33.18	7.40	0.82	96.28	93.30	1.00

4.2.3 Bio-decolorization Process kinetics study of M12C consortium for decolorization of RG-19

The kinetics of decolorization using the developed consortium M12C was modelled using first-order kinetic models for different concentrations of RG-19. The first-order rate law has been used to estimate the rate constants for RG-19 decolorization reactions.

A high degree of linearity (R^2 = 0.90) between ln [C_0/C_t] and time was observed for all the concentrations of RG-19 which implies that the decolorization kinetics could be well fitted to first-order kinetic model described in equation (3.5). First-order kinetics with respect to dye concentration has also been reported by several researchers [201,249]. Effect of initial dye concentration on first order decolorization constant is shown in Figure 4.22. A good decolorization rate was observed in a wide concentration range of RG-19 (25 - 1500 mg/L), however with higher dye concentration beyond 700 mg/L a slight decrease in decolorization rate was observed due to the obvious toxic effect of dye and its degradation metabolites at higher concentrations. Similar results have also been reported by several researchers for decolorization of different azo dyes [135,195,250].

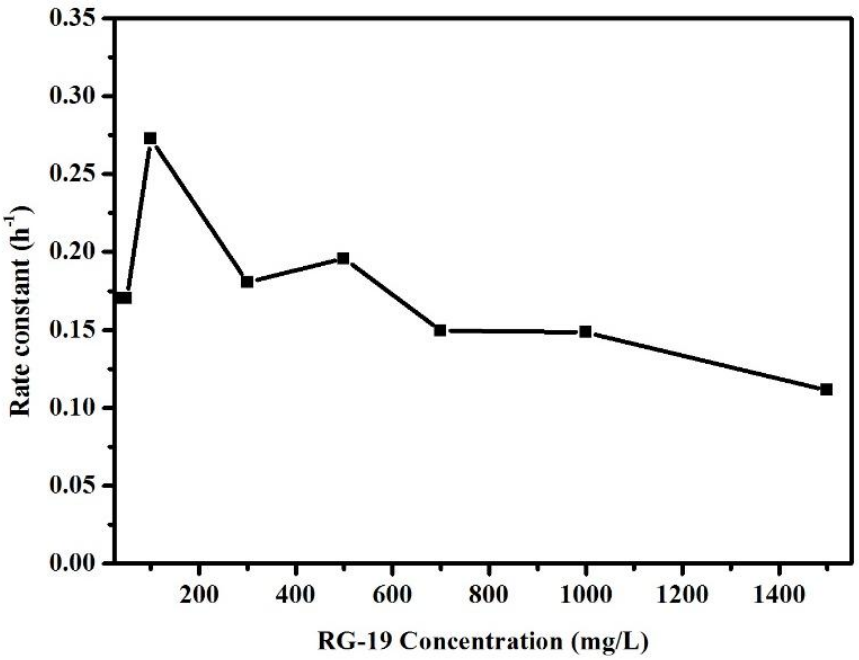


Figure 4.22: Effect of initial dye concentration on decolorization rate constant for RG-19 decolorization using M12C consortium

- **Application of Steady-State Kinetics for The Determination of K_m and V_m**

The developed consortium showed efficient decolorization of RG-19 in a very wide concentration range (i.e. 25 mg/L to 1500 mg/L). The decolorization profile and residual concentration is shown in Figure 4.23. The consortium was found to be highly efficient for removal of RG-19 in wide concentration range.

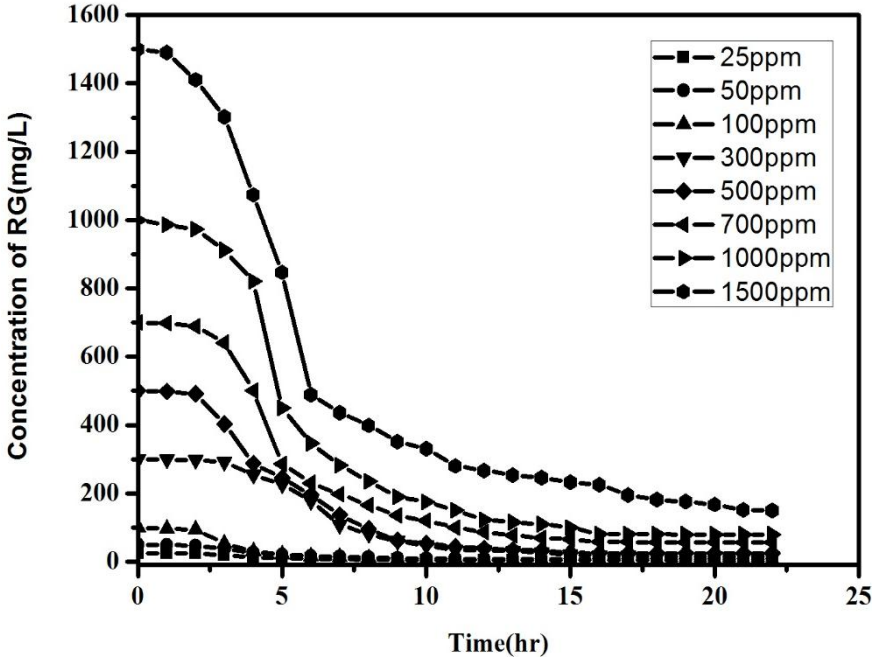


Figure 4.23: Decolorization profile of RG-19 dye at different initial concentration using "M12C" consortium

At lower concentration of 100 mg/L a maximum of 97% decolorization was observed under optimized conditions. The linear plot obtained for decolorization rate against RG-19 concentration (Figure 4.24) depicts that decolorization rate increases with increase in initial dye concentration. This can be explained by the fact that at lower concentration of dye, the efficiency of enzymes to recognise the dye molecules as substrate gets affected which in turn lowers the reaction rate [251].

Michaelis-Menten type rate model, (Equation 3.6) has been used to estimate the maximum decolorization rate and Michaelis-Menten constant for decolorization of RG-19 using M12C consortium. The double reciprocal plot decolorization rate (1/V) against concentration versus (1/S) yields a straight line with $1/V_m$ as the intercept and K_m/V_m as the slope (Figure 4.25). The values obtained for V_m = 120.48 hr^{-1} and K_m = 1674 mg/L.

The maximum decolorization rate, V_{max} was reached, when all the active sites were saturated with RG-19. Maximum value of decolorization rate was achieved, when RG-19 concentration was greater than K_m (1674 mg/L).

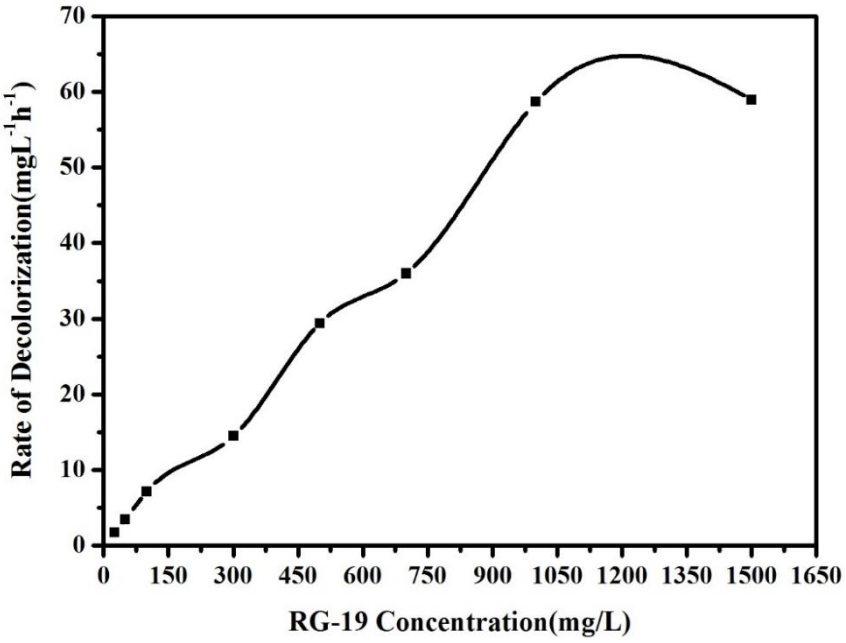


Figure 4.24: Relationship between decolorization rate and RG-19 dye Concentration using "M12C" consortium

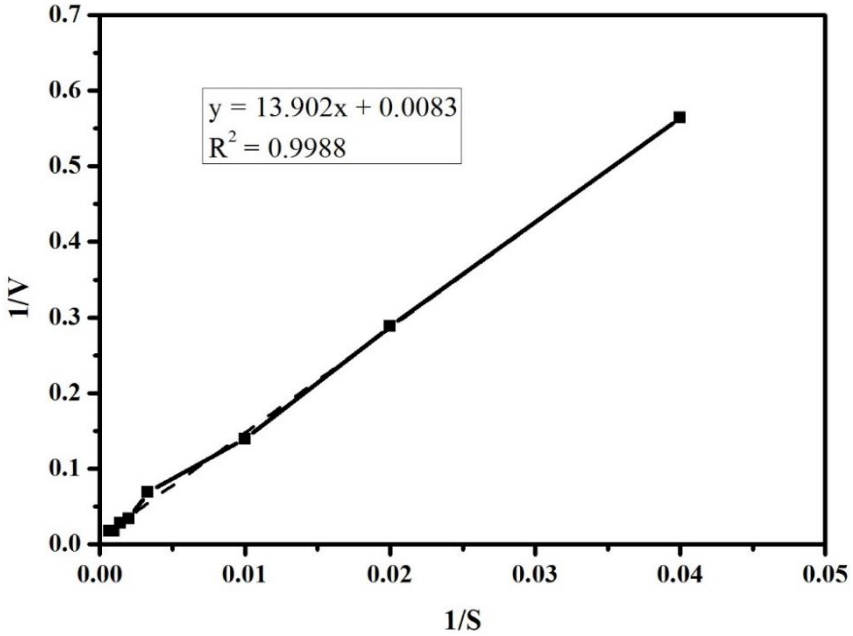


Figure 4.25: Double reciprocal plot of decolorization rates against initial concentration of RG-19 dye using "M12C"

4.2.4 Analysis of Dye Degradation Products of RG-19 Degradation Using M12C Consortium

UV-VIS spectra of control for RG-19 dye before decolorization and samples collected at different time intervals during the process of decolorization using the consortium show noticeable variation in absorption peaks (Figure 4.26). The major peak appeared in the visible region at 635 nm in control dye found to be decreased gradually as the process of decolorization progresses and some new peaks appeared in the UV range. Inspecting the cell mass also showed that microorganisms retained their natural color after decolorization of RG-19 dye. Hence it can be concluded that the decolorization is due to biodegradation of RG-19 dye and is not due to bio-adsorption [193].

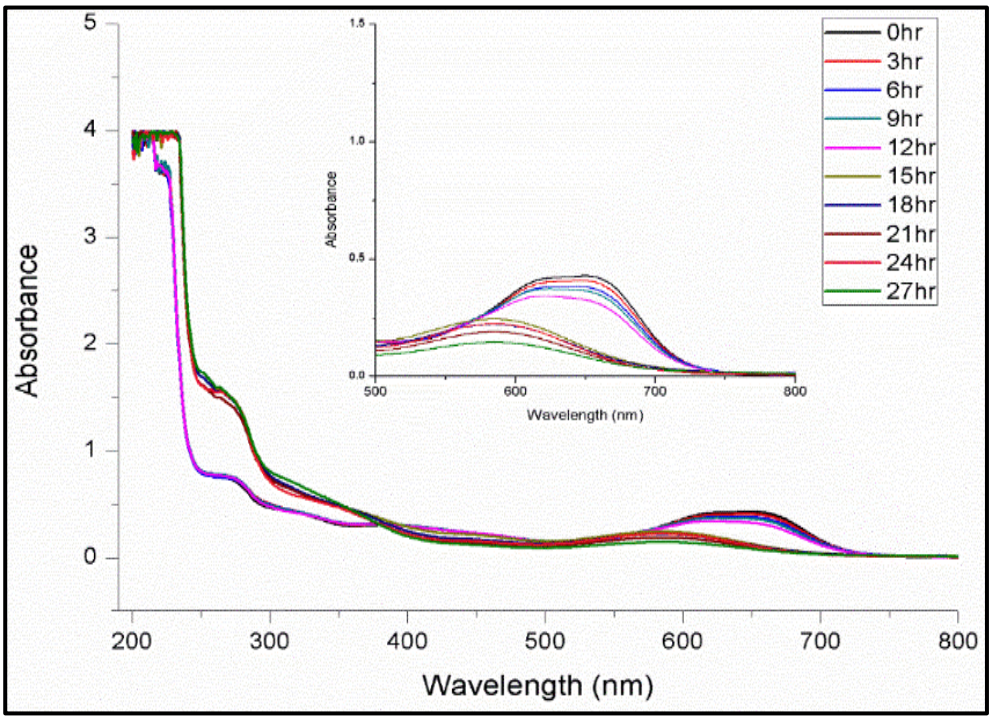


Figure 4.26: UV-VIS spectrum of RG-19 dye decolorization using "M12C"

In HPLC analysis, the control showed single peak at retention time 2.653 min (Figure 4.27) while the chromatogram of metabolites formed after degradation of RG-19 was found to be completely altered. The HPLC chromatogram of metabolites formed after degradation of RG-19 showed disappearance of the major peak which appeared in control dye and appearance of two new major peaks at retention time 2.293 min and 2.607min and two minor peaks at retention times of 2.773 min and 2.900 min. The disappearance

of the single peak and appearance of four new peaks at different retention times supports the mineralization of RG-19 into different metabolites by the developed consortium.

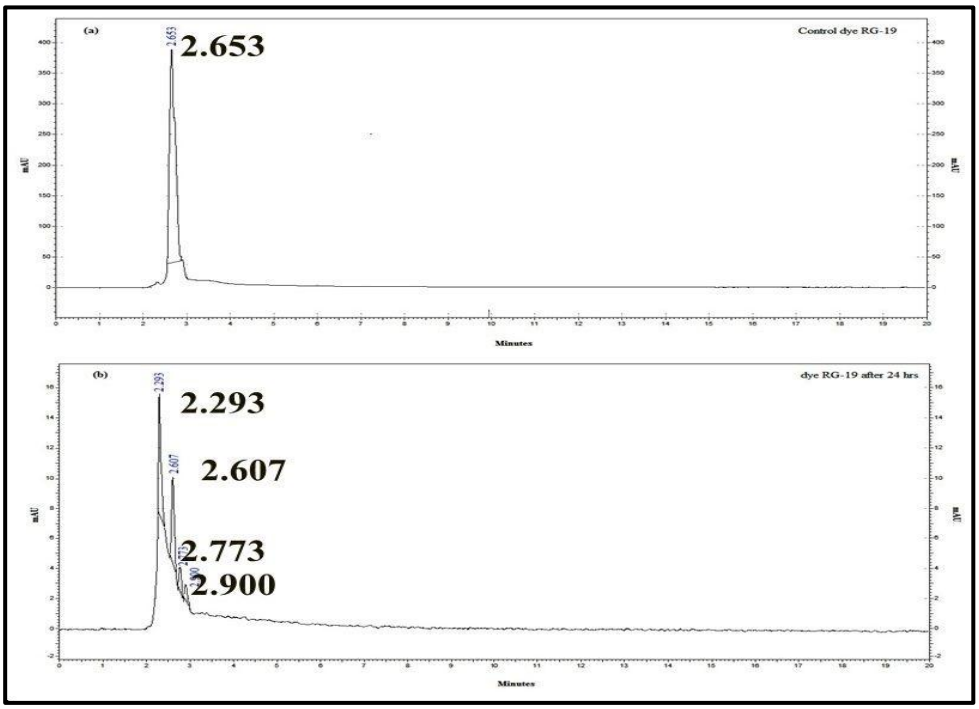
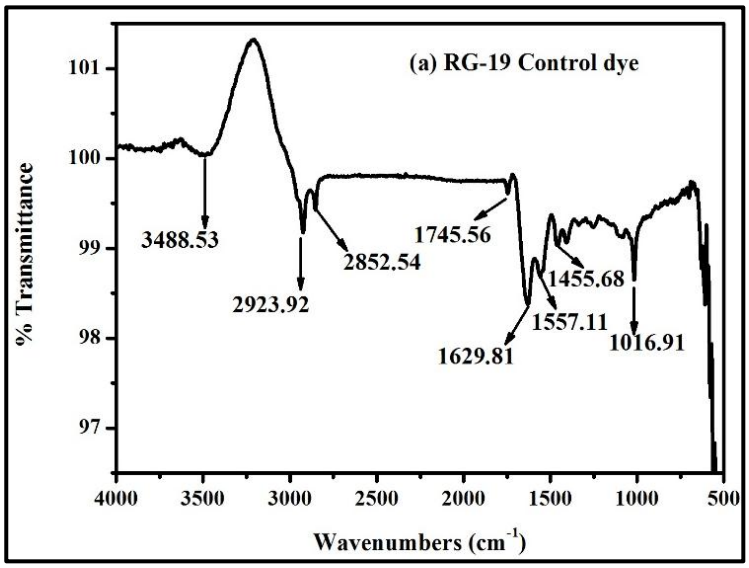


Figure 4.27: HPLC chromatogram for (a) control RG-19 dye (b) and its metabolite obtained after decolorization using consortium "M12C" (after 24 h)

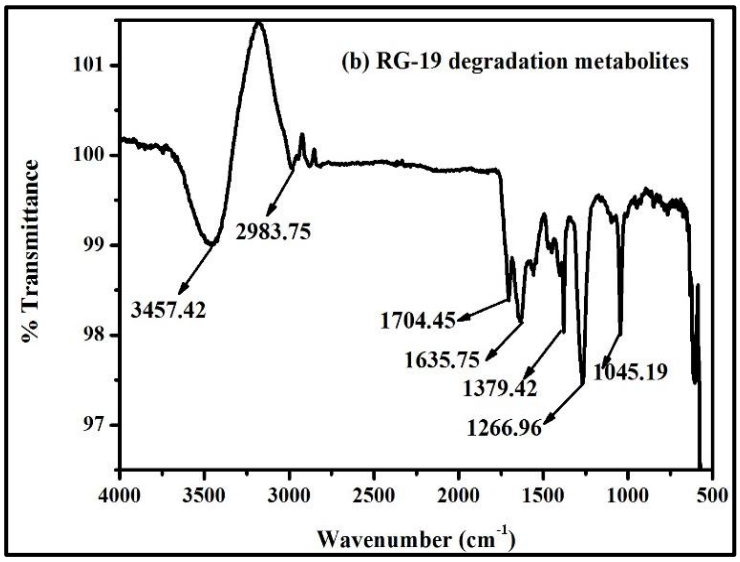
The significant difference in FTIR spectrum of control dye and metabolites formed after decolorization suggests the degradation of RG-19 dye by the developed consortium. Figure 4.28a for FTIR spectra obtained from control of RG-19 dye showed specific peaks at 3488.53 cm^{-1} for N-H stretching vibrations, 2923.92 cm^{-1} for alkanes C-H stretching, 2852.54 cm^{-1} for alkanes C-H stretching, 1745.56 cm^{-1} for C=O stretch, 1629.81 cm^{-1} for N=N stretching in azo group, 1557.11 cm^{-1} for aliphatic nitro compounds, 1455.68 cm^{-1} for C-H bend, 1016.91 cm^{-1} for aliphatic phosphates stretch.

The metabolites obtained after decolorization of RG-19 dye Figure 4.28b showed some new peaks at 3457.42 cm^{-1} for primary amines, 2983.75 cm^{-1} for C-H stretching of alkanes, 1704.45 cm^{-1} for C=O stretch of ketone, 1635.75 cm^{-1} for NH bend of primary amines, 1379.42 cm^{-1} for nitrate ion, 1266.96 cm^{-1} & 1045.19 cm^{-1} for C-N stretch of aromatic primary amine. The disappearance of peak at 1629.81 cm^{-1} and appearance of some new peaks in the FTIR spectra of sample after decolorization gives the evidence of azo bond cleavage and formation of different metabolites [192,252]. The formation of

aromatic amines on decolorization can be due to the reductive cleavage of azo bond (-N=N-).



(a)



(b)

Figure 4.28: FTIR spectrum of (a) control RG-19 dye (b) and its metabolite obtained after RG-19 decolorization using consortium "M12C" (after 24 h)

GC-MS analysis of the metabolites obtained after degradation confirms the production of low molecular weight compounds through the asymmetric cleavage of RG-19 by the developed consortium. Mass spectrum analysis and NIST library, suggests proposed

metabolites to be identified as long chain aliphatic hydrocarbons, unsaturated carboxylic acids and organic sulphides (Figure 4.29).

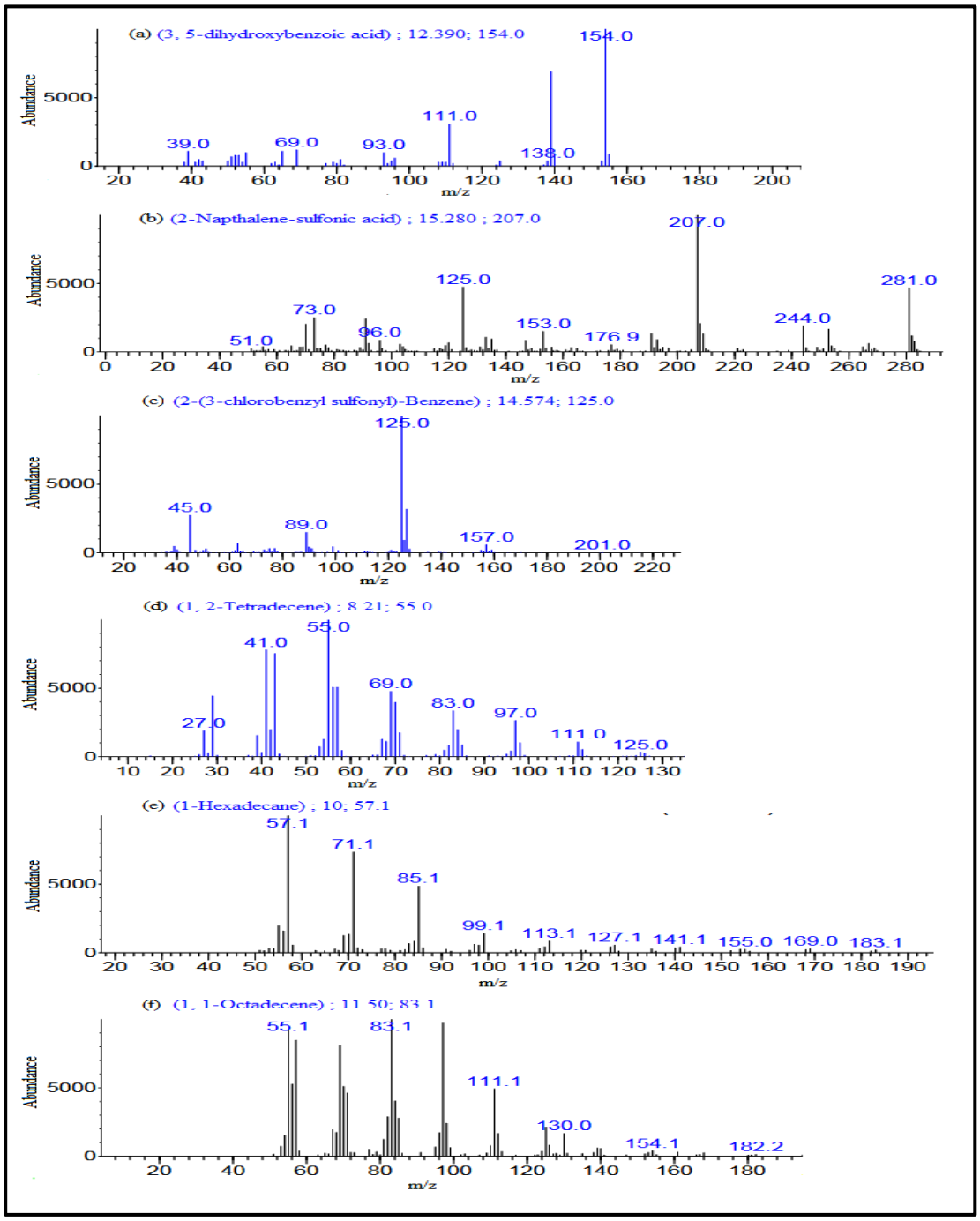


Figure 4.29: GC-MS of RG-19 dye after decolorization using "M12C" (after 24 hr) (a) 3, 5-dihydroxybenzoic acid (b) 2-Napthalene-sulfonic acid (c) 2-(3-chlorobenzyl sulfonyl)-Benzene (d) 1, 2-Tetradecene (e) 1-Hexadecane (f) 1, 1-Octadecene

The unsaturated carboxylic acid isomer might be (3, 5-dihydroxybenzoic acid) at retention time of 12.390 min and mass peak of 154. Organic sulphides obtained from decolorization of RG-19 are supposed to be (2-Napthalene-sulfonic acid) with a retention time of 15.280 min; mass peak of 207 and (2-(3-chlorobenzyl sulfonyl)-Benzene) at retention time 14.574 min; mass peak of 125. Aliphatic hydrocarbon obtained are namely (1, 2-Tetradecene) at retention time 8.21min; mass peak of 55, (1-Hexadecane) at retention time 10 min; mass peak of 57.1 and (1, 1-Octadecene) at retention time 11.50 min mass peak of 83.1. This implies the recalcitrant RG-19 has been converted into simple metabolizable structures.

4.2.5 Optimization of Decolorization Potential of "MA12C" Consortium for The Decolorization of Mixture of Dyes Using Response Surface Methodology

As discussed in section 4.2.1, decolorization efficiency of consortium "MA12C" was found to be slightly higher than consortium "M12C". The second part of the study primarily focused on the dye decolorization capacity of "MA12C" consortium for SDM consisting of three structurally different industrially important textile dyes i.e. RG-19, RR-198 and RNB. Since color industry wastewater often contains mixtures of different azo dyes with variety of chemical structures, it is necessary to focus on finding ways to treat mixture of dyes under industrially relevant conditions. To make this process industrially feasible, it becomes necessary to study the synergistic effect of different important physico-chemical parameters on the decolorization efficiency of the developed consortium. An attempt has been made for the optimization of the process parameters using Response surface methodology to enhance the dye decolorization efficiency of "MA12C" for efficient decolorization of SDM. Decolorization kinetics of SDM at different concentration was studied at optimized condition and the kinetic parameters have been estimated.

- **Batch Decolorization Study to Evaluate Individual Effect of Different Physico-Chemical Parameters on Dye Decolorization Efficiency of "MA12C"**

To maximize the decolorization efficiency of the developed consortium and to evaluate the effect of different process parameters, batch decolorization experiments were

conducted for optimization of inoculum volume, pH, incubation temperature, carbon source, nitrogen source etc. by changing one factor at a time and keeping other factors constant.

To evaluate the need for carbon and nitrogen source, experiments were conducted with different combination of carbon and nitrogen source. As discussed earlier in section 4.2.2, glucose and yeast extract were chosen as suitable carbon and nitrogen source respectively for this study. Effect of only glucose, combined effect of glucose and yeast extract and effect of only yeast extract on decolorization efficiency of "MA12C" has been studied. As evident from Figure 4.30, presence of carbon source like glucose has very negligible effect, however presence of yeast extract in the medium was found to be very essential for decolorization of SDM using the developed consortium. In presence of only glucose as co-substrate, only 9.4% decolorization has been achieved, however with yeast extract as the only co-substrate in the medium gives 95.7% decolorization of SDM. Use of both glucose and yeast extract as the co-substrates, the decolorization efficiency of the consortium was found to be increased slightly by 2.7% which is very negligible. Hence, further studies were conducted with yeast extract as the only co-substrate.

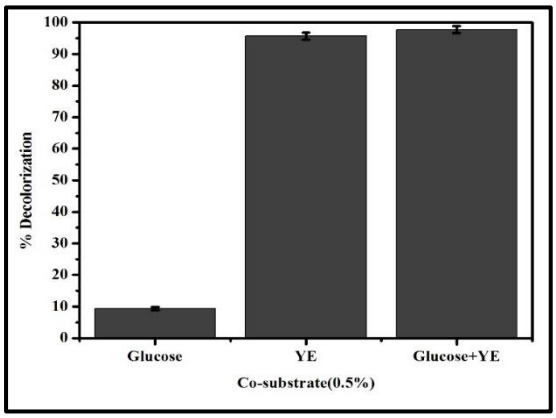


Figure 4.30: Effect of Carbon and Nitrogen source on decolorization efficiency of "MA12C"

As yeast extract was found to be the only co-substrate required by the consortium "MA12C" for efficient decolorization of SDM, it becomes necessary to explore the optimum amount of the same required for achieving maximum decolorization potential. Varying the amount of YE in the culture medium from 0.1 g/100mL to 2.5 g/100mL, it has been observed that (see Figure 4.31), increase in YE concentration has positive effect on decolorization efficiency of the consortium and highest decolorization of SDM

(i.e.96.24%) has been observed at 1.5 g/100mL of YE concentration. However, further increasing the YE amount was found to have negative effect on decolorization of SDM.

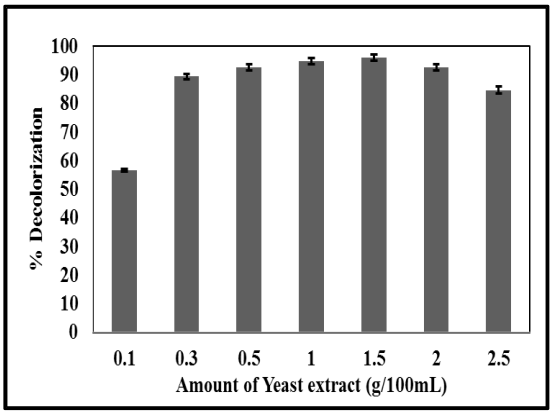


Figure 4.31: Effect of Yeast extract on decolorization efficiency of "MA12C"

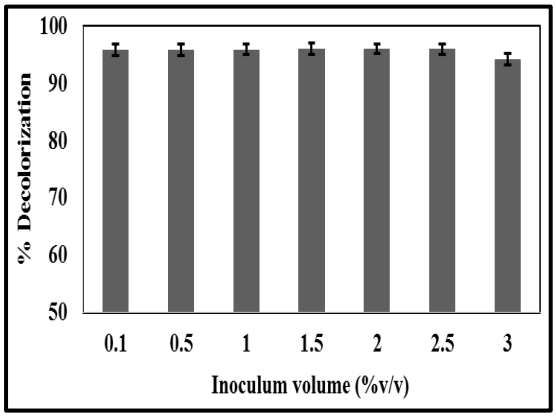


Figure 4.32: Effect of inoculum volume on decolorization efficiency of "MA12C"

To explore the effect of culture inoculum volume on decolorization of SDM, 100 mL of microbial culture media containing 100 mg/L concentration of SDM has been inoculated with different inoculum volume (0.1% (v/v) to 3% (v/v)). It can be concluded from Figure 4.32 that, change in inoculum volume has no apparent effect on the decolorization efficiency of the consortium. Almost 96% of decolorization has been achieved with all tested inoculum volumes, hence all the experiments were performed with 1% (v/v) inoculum volume as reported by many researchers.

Textile industry wastewater has been reported to contain 15-20% of salt concentration [84,247]. A slight decrease in dye decolorization efficiency of the developed consortium has been observed with increasing the salt concentration (in the form of NaCl) in the medium (see Figure 4.33). Up to 6% (w/v) NaCl concentration, the consortium was able

to achieve 94-96% decolorization of SDM. With further increase in salt concentration, a decrease in color removal efficiency has been observed and at 15% (w/v) NaCl concentration, maximum of 85.63% decolorization of SDM has been achieved. The ability of the consortium to achieve significant amount of decolorization of SDM up to 15% (w/v) NaCl concentration signifies it's potential to be used for efficient decolorization of textile effluents.

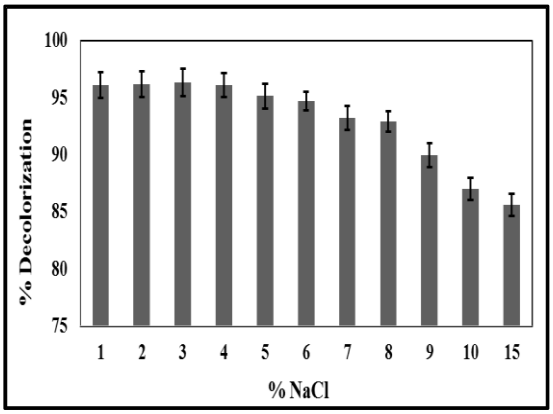


Figure 4.33: Effect of salinity on decolorization efficiency of "MA12C"

Incubation temperature is a major aspect of any kind for bioremediation process as it affects microbial growth and enzyme activity. As wastewater released from textile processing units are having high temperature, hence it becomes an important aspect to know the optimal temperature range of microorganisms used in bioremediation of dye effluent. Batch decolorization experiments were conducted with varying incubation temperature (20 $^{\circ}$C – 45 $^{\circ}$C) to study the effect of temperature on decolorization efficiency of the consortium "MA12C". Maximum decolorization efficiency of the developed consortium has been observed between 30°C -35°C (see Figure 4.34). However increase or decrease of temperature beyond this range the decolorization rate found to be decreased gradually.

Effect of pH on decolorization efficiency of "MA12C" has been studied and the results are shown in Figure 4.35. The developed consortium showed efficient decolorization between pH 7 to pH 9 and highest decolorization of 97% was achieved at pH 8. Our developed consortium was able to decolorize SDM in a wide pH range, however it is found to be more efficient in basic pH as compared to acidic pH.

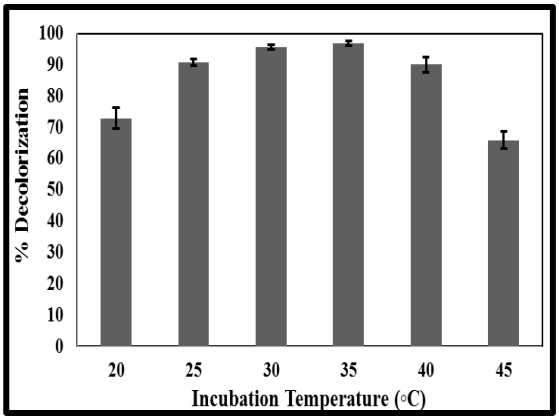


Figure 4.34: Effect of incubation temperature on dye decolorization efficiency of "MA12C"

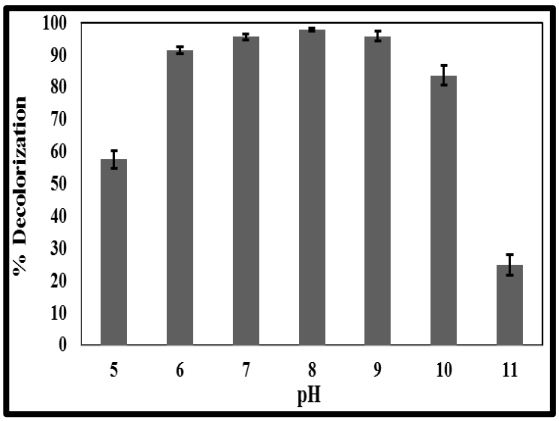


Figure 4.35: Effect of pH on decolorization efficiency of "MA12C"

Hence from the above study it has been concluded that, pH, incubation temperature and concentration of Yeast extract in the medium were the important parameters affecting the dye decolorization efficiency of the developed consortium "MA12C". To study the combined effect of these three factors on the dye decolorization process, optimization through response surface methodology using Box-Behnken analysis has been used.

- **Box-Behnken analysis**

In this study, three independent variables or factors were chosen to maximize the decolorization efficiency of the developed consortium "MA12C" including Yeast extract concentration (YE), incubation temperature and pH. Table 3.3 shows the experimental parameters and the experimental Box–Behnken design levels used which was selected based on preliminary experiments.

In order to study the synergic effect of these factors, 17 experimental runs (consisting of 12 trials plus 5-centre points) were carried out to obtain a quadratic model for decolorization of the SDM with a developed microbial consortium "MA12C" according to the Box-Behnken design. Each run was performed in triplicate and mean values for experimental data of % decolorization SDM are presented in Table 4.8 where the predicted values of response were obtained from quadratic model fitting techniques.

The experimental results were analysed through RSM to obtain an empirical model for the best response. The quadratic model was used to explain the mathematical relationship between the independent variables and dependent responses. The regression model equation (in uncoded form) showing the effect of all three independent variable including interaction effect on decolorization of SDM can be presented as below:

$$\boldsymbol{Y = +92.52 - 7.14A + 11.50B + 13.31C + 3.55AB - 3.75AC + 3.48\,BC - 18.95\,A^2 - 19.69B^2 - 15.51C^2} \quad (4.2)$$

Where Y is the predicted response and A, B, C are the coded values of the independent variables incubation temperature (°C), pH and concentration of yeast extract (g/100 mL) respectively.

- **Model Adequacy**

Linear, interactive, quadratic and cubic models were fitted to the experimental data to obtain the regression equations. To decide the model adequacy to represent SDM decolorization using "MA12C" consortium, all the models were analyzed with Sequential model Sum of Squares, Model Summary and Statistics Lack of Fit Test (Table-4.9). The Lack of fit *P*-value for quadratic model larger than 0.05 suggests that the model correctly explains the relationship between the factors and the response [239–241]. Cubic model was found to be aliased. In Model summery statistics also cubic model was aliased while quadratic model showing the maximum "Adjusted R-Squared" and the "Predicted R-Squared" values. The quadratic model also produces the least PRESS among all other models which suggests to choose it for further analysis.

Table 4.8: Three factor Box-Behnken design with experimental as well as predicted responses of dependent variable (% of decolorization of SDM) using microbial consortium "MA12C"

Runs	Factors						Response		Internally Studentized Residual
	Coded values			Actual values			% of decolorization		
	A	B	C	A	B	C	Observed	Predicted	
1	-1	-1	0	20	5	1.3	52.30	53.07	-0.728
2	0	0	0	32.5	7.5	1.3	94.20	92.52	0.883
3	0	0	0	32.5	7.5	1.3	92.00	92.52	-0.273
4	+1	0	+1	45	7.5	2.5	62.12	60.48	1.542
5	-1	0	-1	20	7.5	0.1	46.50	48.14	-1.542
6	0	0	0	32.5	7.5	1.3	90.80	92.52	-0.904
7	0	0	0	32.5	7.5	1.3	93.50	92.52	0.515
8	0	+1	+1	32.5	10	2.5	83.20	85.61	-2.270
9	+1	-1	0	45	5	1.3	30.50	31.68	-1.109
10	0	-1	+1	32.5	5	2.5	55.20	55.66	-0.432
11	0	-1	-1	32.5	5	0.1	38.40	35.98	2.270
12	-1	+1	0	20	10	1.3	70.15	68.97	1.109
13	+1	+1	0	45	10	1.3	62.57	61.80	0.728
14	0	0	0	32.5	7.5	1.3	92.10	92.52	-0.221
15	+1	0	-1	45	7.5	0.1	40.12	41.35	-1.161
16	-1	0	+1	20	7.5	2.5	83.50	82.26	1.161
17	0	+1	-1	32.5	10	0.1	52.50	52.04	0.432

Table 4.9: Adequacy of the model for SDM decolorization using "MA12C"

Source	Sum of Squares	*df*	Mean Square	F Value	p-value Prob > F	Remarks
Sequential model sum of squares						
Mean versus Total	76401.47	1	76401.47			
Linear versus Mean	2884.36	3	961.45	2.59	0.0975	
2FI versus Linear	155.10	3	51.70	0.11	0.9519	
Quadratic versus 2FI	4638.98	3	1546.33	341.55	<0.0001	Suggested
Cubic versus Quadratic	24.50	3	8.17	4.55	0.0888	Aliased
Residual	7.19	4	1.80			
Total	84111.61	17	4947.74			
Source	**Sum of Squares**	***df***	**Mean Square**	**F Value**	**p-value Prob > F**	**Remarks**
Lack of Fit Tests						
Linear	4818.59	9	535.40	297.94	<0.0001	
2FI	4663.48	6	777.25	432.52	<0.0001	
Quadratic	24.50	3	8.17	4.55	0.0888	Suggested
Cubic	0	0				Aliased
Pure Error	7.19	4	1.80			
Source	**Std. Dev.**	**R-Squared**	**Adjusted R-Squared**	**Predicted R-Squared**	**PRESS**	**Remarks**
Model Summary statistics						
Linear	19.27	0.3741	0.2297	0.0968	6963.94	

2FI	21.61	0.3942	0.0307	-0.4207	10953.45	
Quadratic	2.13	0.9959	0.9906	0.9477	403.28	Suggested
Cubic	1.34	0.9991	0.9963		+	Aliased

+ Case(s) with leverage of 1.0000: PRESS statistic not defined

- **Fitting of Second-Order Polynomial Equation and Statistical Analysis**

Analysis of variance (ANOVA) was used to statistically analyze the experimental data for the decolorization of SDM using the "MA12C" consortium and the result is shown in Table-4.10. The high *F*-value 188.45 and corresponding p-value < 0.0001 obtained from the ANOVA quadratic polynomial model, show the high significance of the model, indicating there is only 0.01% chance that the model *F*-value this large could occur because of noise. The lack of fit *F*-value of 4.55 is not significant as the corresponding *p*-value 0.0888 is larger than 0.05 which suggests a good fit of the model. The linear terms (A, B and C) and three 2FI terms (AB, BC and AC) turns out to be significant as their p-values are less than 0.05. This implies that the interaction effects of independent variables with each other affects the decolorization efficiency of "MA12C" consortium. P-value << 0.05 for the quadratic terms indicates that there is curvature in the response surface and the relationship of % decolorization of SDM using the "MA12C" consortium with incubation temperature, broth pH and yeast extract concentration followed a curved line rather than a straight line. Hence from ANOVA statistical analysis it can be concluded that, all the independent variables significantly affects the decolorization efficiency of the consortium.

Table 4.10: Summary of analysis of variance results; ANOVA for response surface quadratic model of % of Decolorization using microbial consortium "MA12C"

Factors	**Sum of Squares**	**Degree of Freedom**	**Mean Square**	**F Value**	**p-value Prob > F**	
Model	7678.45	9	853.16	188.45	< 0.0001	significant
A-Temperature	408.12	1	408.12	90.15	< 0.0001	

B-pH	1058.46	1	1058.46	233.79	< 0.0001	
C-Yeast Extract	1417.78	1	1417.78	313.16	< 0.0001	
AB	50.55	1	50.55	11.17	0.0124	
AC	56.25	1	56.25	12.42	0.0097	
BC	48.30	1	48.30	10.67	0.0137	
A^2	1512.41	1	1512.41	334.06	< 0.0001	
B^2	1631.99	1	1631.99	360.47	< 0.0001	
C^2	1012.56	1	1012.56	223.65	< 0.0001	
Residual	31.69	7	4.53			
Lack of Fit	24.50	3	8.17	4.55	0.0888	not significant
Pure Error	7.19	4	1.80			
Correlation Total	7710.14	16				

Table 4.11: Statistical summary of quadratic model for decolorization of SDM

Standard Deviation	2.13	R-Squared	0.9959
Mean	67.04	Adj. R-Squared	0.9906
C.V. %	3.17	Pred. R-Squared	0.9477
PRESS	403.28	Adeq. Precision	37.281

A high R^2 value (0.9959) shows a high degree of correlation between the observed and the predicted responses. The predicted R^2 of 0.9477 is in good agreement with the adjusted R^2 of 0.9906 representing adequacy of the model. The adequate precision value of 37.281 indicates adequate signal. The C.V % of 3.17 and low standard deviation of 2.13 indicates that the model can be effectively used to predict the decolorization of SDM in the experimental range (Table 4.11).

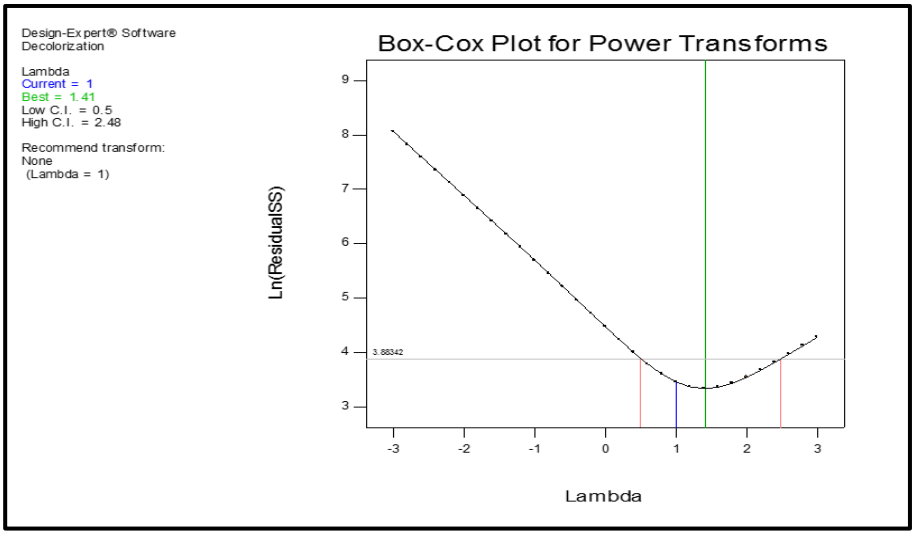


Figure 4.36: Box-Cox Plot of Power Transforms for percentage of decolorization using microbial consortium "MA12C"

Figure 4.36 shows the Box-Cox plot of power transforms for % decolorization of SDM using the "MA12C" consortium. The minimum confidence and maximum confidence interval for the model are 0.5 and 2.48 respectively and the value of current point of C.I. ($\lambda = 1$) falls closest to the best lambda value of 1.41 and is within the C.I. which indicates no transformation of model is required.

The steep slope or curvature in the perturbation graph plot (Figure 4.37) indicates that, the response is sensitive towards each variables [239,241]. The % decolorization of SDM found to be changed with gradual change in values of individual factors from low (-1) to high (+1).

As explained earlier in section 4.2.2, the normal probability plot of the residuals explain, whether the assumptions that errors are normally distributed and are independent of each other, and that the error variance is homogeneous are true [245]. The residuals also provides information regarding the lack of fit of the selected model [119]. A straight line in the residual plot (Figure 4.38) indicates that there is no serious violation of assumptions underlying the analysis and it confirmed the normality assumptions and independence of the residuals [240].

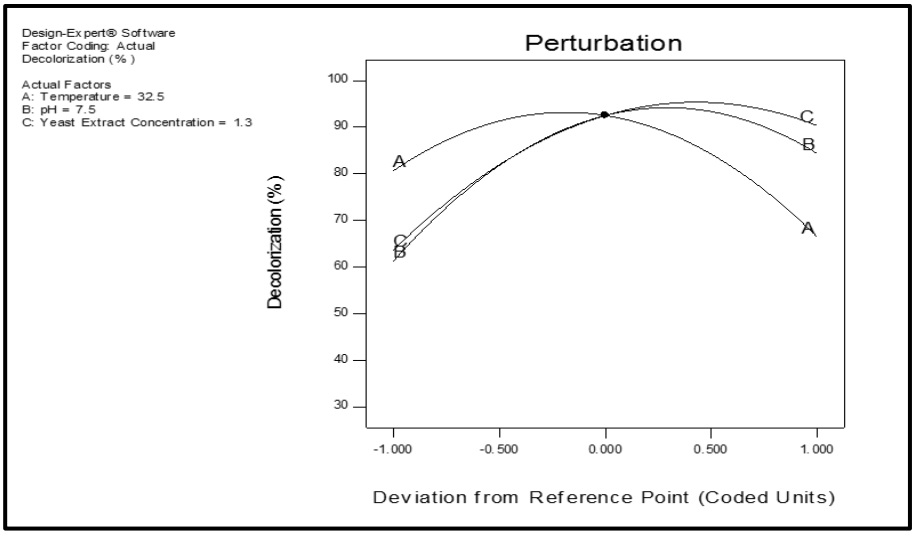


Figure 4.37: Perturbation graph showing the effect of each of the independent variables on percentage of decolorization while keeping other variables at their respective mid-point levels for decolorization of SDM using consortium "MA12C"

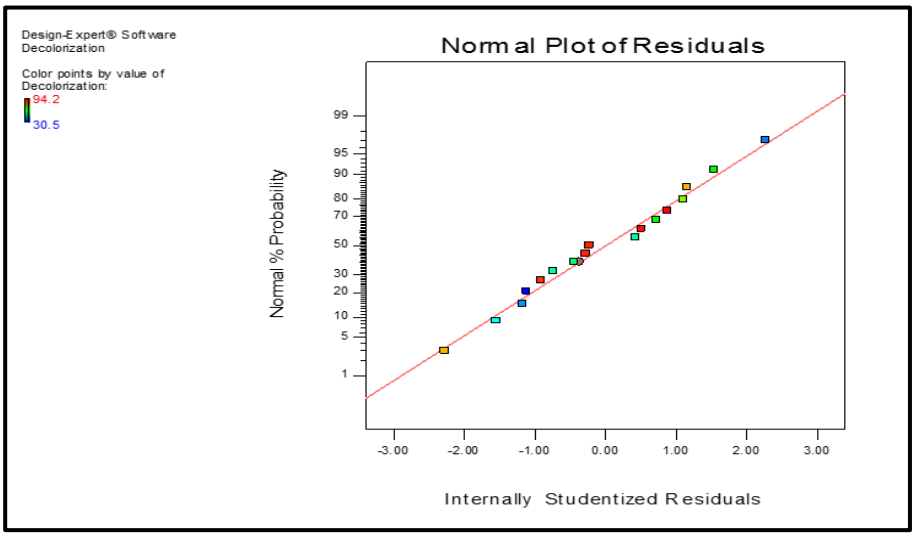


Figure 4.38: Normal plot of residuals showing the relationship between normal probability (%) and internally studentized residuals for decolorization of SDM using consortium "MA12C"

Figure 4.39 shows the relationship between predicted and actual values of response. Less deviation of the point cluster around the diagonal line signifies a good model fit [246]. In the Residual vs. Run plot (Figure 4.40) as there is no point far below the lower critical line, it can be concluded that the model fits the observation very well [239].

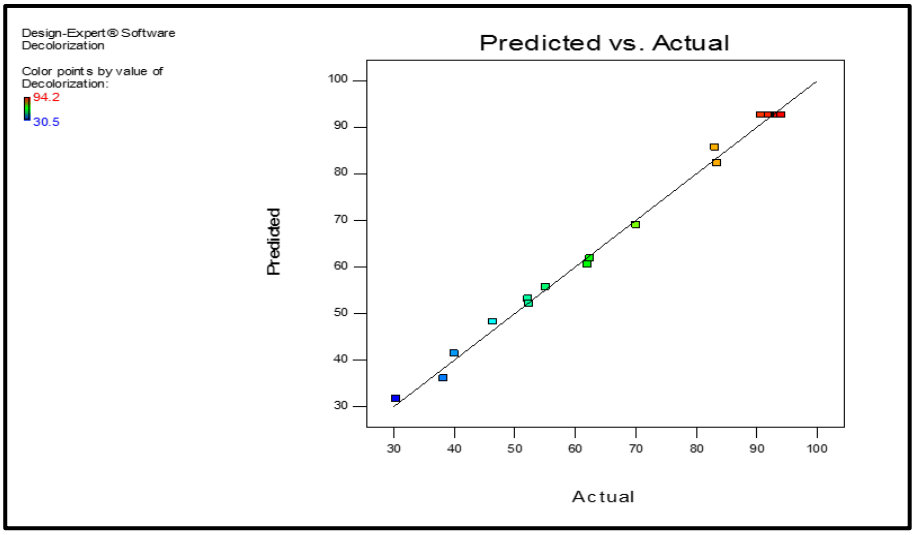


Figure 4.39: Plot showing the correlation between the predicted and actual experimental values for decolorization of SDM using "MA12C"

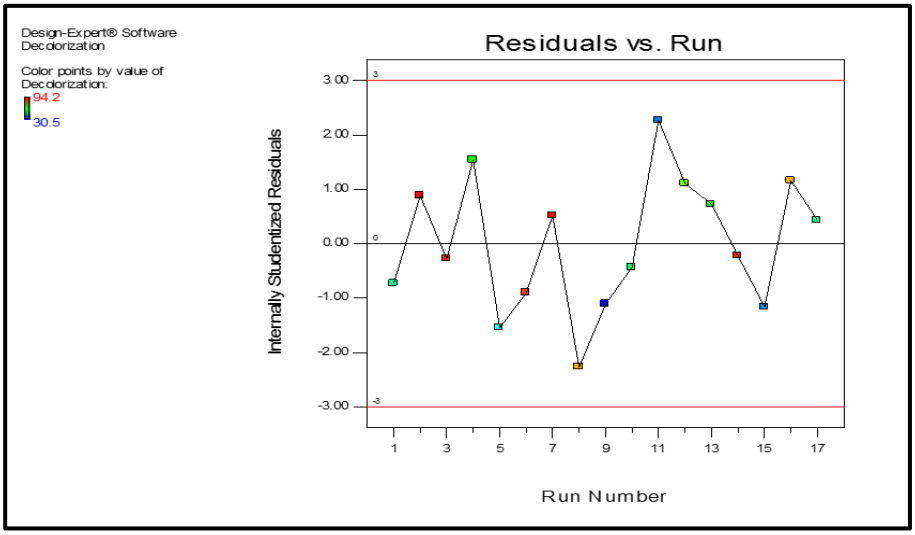


Figure 4.40: Plot of residuals versus the experimental run order of model for decolorization of SDM using "MA12C" consortium

- **Effect of Various Parameters On SDM Removal Efficiency of MA12C Consortium**

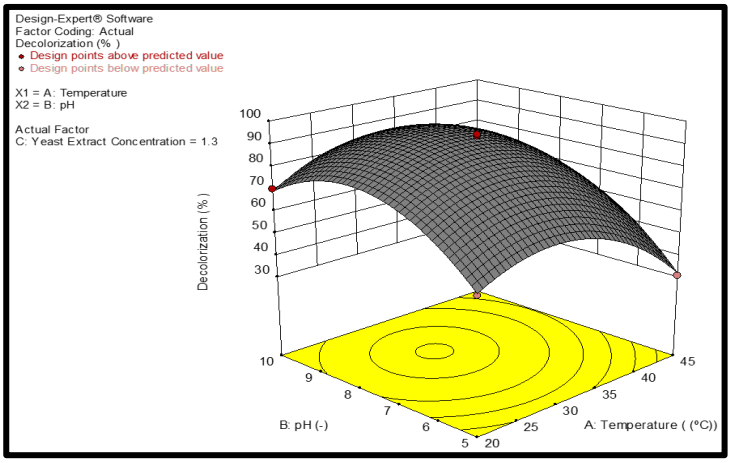
The individual and interaction effects of independent variables on the response can be studied from the three-dimensional response surface plots and two-dimensional contour plots. Varying two variables within their studied ranges and keeping the third variable constant to the zero level, gives a better understanding about the effects of process

variables on the response. Hence from these plots the optimum values of the parameters can be determined to maximize the response.

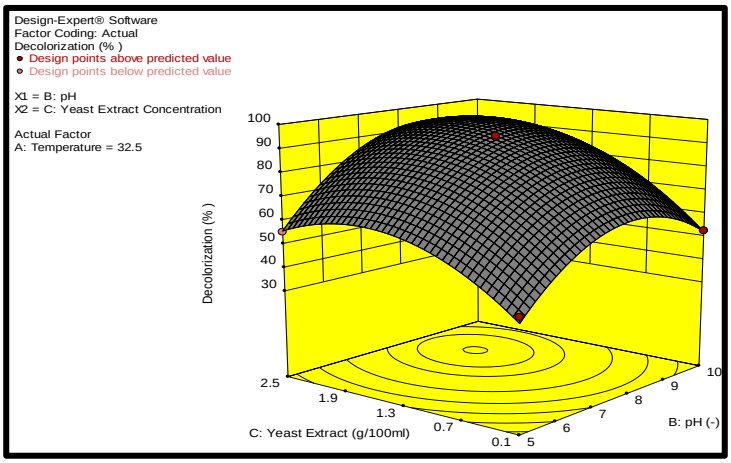
The effect of the three independent factors (incubation temperature, pH and yeast extract concentration) on % Decolorization of SDM using “MA12C” consortium are represented through 3D surface plots and their corresponding contour plots (Figure 4.41). As explained earlier, elliptical shape of contour plot indicates a prominent interaction, where as a circular contour plot indicates a negligible effect [240].

Figure 4.41a shows the combined effect of incubation temperature and medium pH on % decolorization of SDM at constant yeast extract concentration of 1.3 g/100mL. It is evident from the figure that, with increase in temperature, decolorization efficiency increased and maximum decolorization of SDM was achieved in the range of 30 °C to 35 °C. A decrease in decolorization efficiency was noticed with further increase in temperature. This indicates that like “M12C”, “MA12C” consortium is also highly efficient in mesophilic temperature range and at higher temperature, organisms either die or becomes inactive which leads to decreased decolorization potential. Effect of pH on decolorization efficiency of the consortium shows that, maximum decolorization was achieved in slightly alkaline pH in the range of 7.5-8.5. Increasing or decreasing the pH beyond this range leads to decrease in percentage decolorization of SDM. The elliptical shape of the contour plot signifies a significant quadratic effect of temperature and pH only in the mid-range of parameters for decolorization of SDM using “MA12C” consortium.

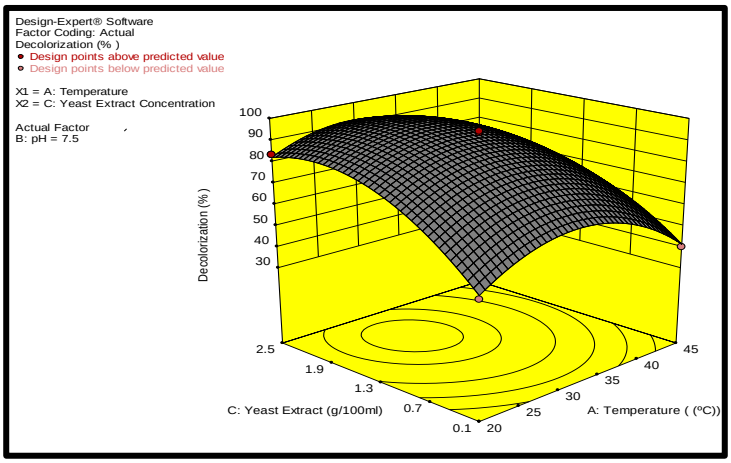
Figure 4.41b explains the surface plot of response for the combined effect of pH and YE concentration on decolorization efficiency of “MA12C” strain. With increase in yeast extract concentration, decolorization efficiency was found to be increased which signifies the effective use of yeast extract for regeneration of NADH which is vital for the reduction of azo bonds. Synergistic effect of pH and yeast extract was indicated by the curvature in the surface plot at higher yeast extract concentration and slightly alkaline pH range.



(a)



(b)



(c)

Figure 4.41: Three-dimensional response surface plot for the effect of (a) pH and Temperature (b) Yeast Extract and pH (c) Yeast Extract and Temperature on percentage of SDM decolorization using microbial consortium

The three dimensional surface plot of temperature and yeast extract concentration (Figure 4.41c) shows that, in mesophilic temperature range, increase in yeast extract concentration imparts positive effect on decolorization efficiency of the consortium.

Studying the synergistic effects of all the independent variables, it can be concluded that, consortium "MA12C" follows the similar trend as consortium "M12C". However, an increased efficiency of decolorization has been achieved for "MA12C" consortium as compared to "M12C".

- **Model validation and confirmation**

The optimized combination of variables obtained from RSM study were validated through some confirmatory experiments. Validation experiments were performed at the predicted conditions and the results were compared with the predicted responses. The optimal combination of variables obtained through this study was temperature 29.909 °C, pH 8.290 and yeast extract concentration 1.888 g/100mL. Three validation experiments were performed under the optimized condition and the average color removal efficiency achieved was 97.95% which is very close to the predicted value i.e. 98.341%. Hence, a good agreement between the predicted and experimental results validate the model and it concludes that RSM is an effective tool for optimizing the process to achieve maximum decolorization efficiency of the developed consortium for the decolorization of SDM.

4.2.6 Study of Biodecolorization Kinetics of SDM Using "MA12C" Consortium Under Optimized Condition

Dye decolorization efficiency of microorganisms were found to be greatly influenced by initial dye concentration in the medium. At lower dye concentrations, efficiency of bacterial enzymes to recognize dye molecule decreases and toxic effect of dye at higher concentrations also exert negative effect on microbial growth. Hence it becomes necessary to study the dye concentration range suitable for efficient color removal using the developed consortium. As can be depicted in Figure 4.42 and Figure 4.43, our developed consortium "MA12C" is highly efficient in dye decolorization and achieves 88%- 98% removal of SDM in a wide concentration range (25 mg/L to 1500 mg/L) at optimized conditions. At lower dye concentrations (50 mg/L & 100 mg/L), the consortium was able to achieve upto 98% color removal within 14h of incubation, which

gradually found to be decreased when the dye concentration has been increased to 1500 mg/L due to the obvious toxic effect of the dye molecules.

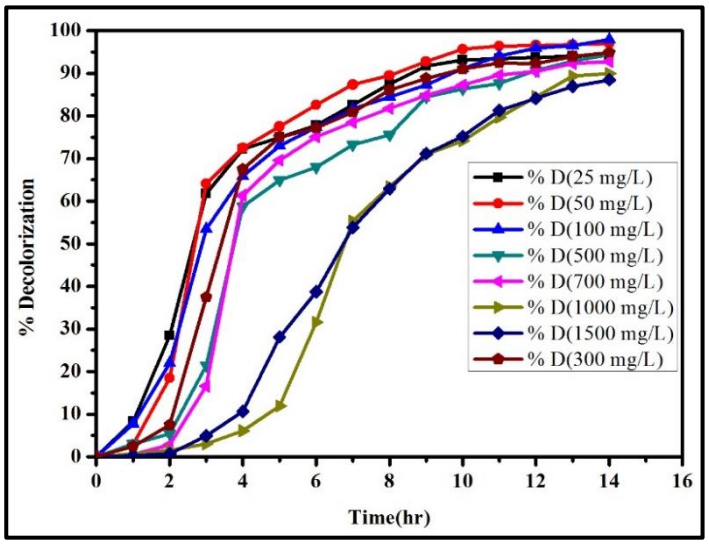


Figure 4.42: Effect of SDM concentration on decolorization efficiency of "MA12C" strain

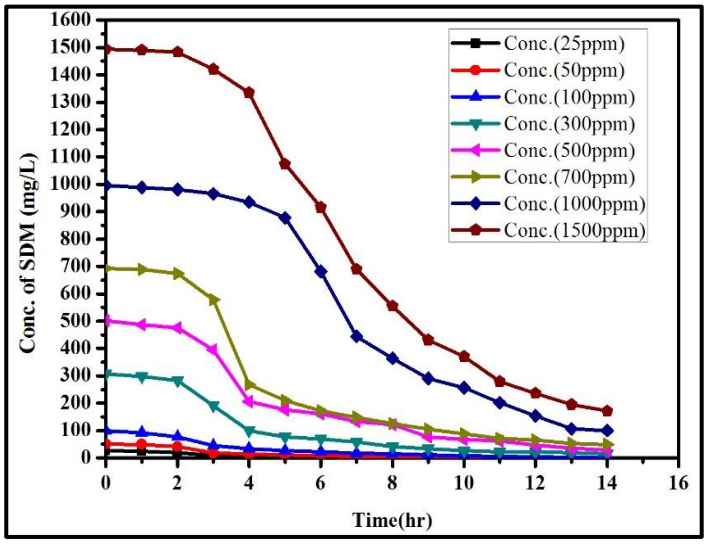


Figure 4.43: Dye degradation profile at different initial SDM concentrations using "MA12C" consortium

A high degree of linearity (R^2 = 0.90) between the ln [C_0/C_t] and time was observed for all the concentrations of SDM which implies that the decolorization kinetics of SDM removal using MA12C consortium could be well fitted to first-order kinetic model described in equation (3.5). Figure 4.44 depicts the relationship between First order rate constant and SDM initial concentration. With increase in dye concentration, the value of decolorization rate constant was found to be decreased, this may be attributed to the fact

that, at higher dye concentrations microbial growth gets depressed due to the obvious toxic effect of dye molecules which affects the decolorization efficiency of organisms.

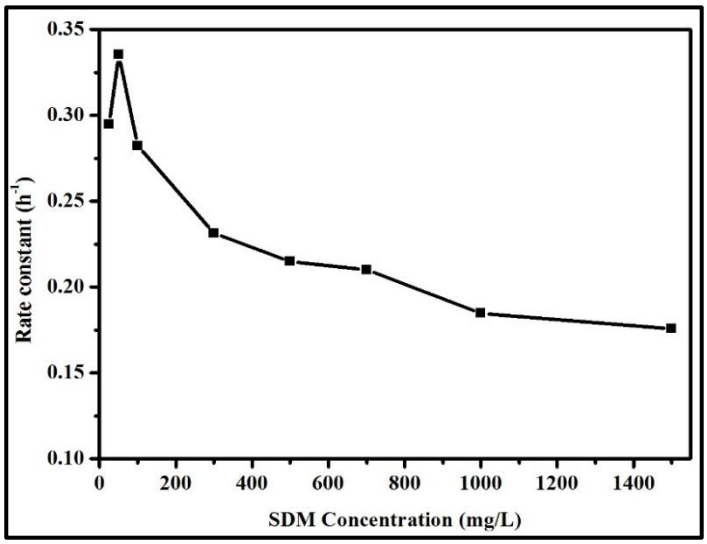


Figure 4.44: Effect of initial dye concentration on decolorization rate constant for SDM decolorization using MA12C consortium

- **Application of Steady-State Kinetics for The Determination of K_m and V_m**

Michailis-Menten rate model has been used to estimate the kinetic parameters of SDM decolorization using MA12C consortium (Equation 3.6). Michalis constant, K_m and the maximum decolorization rate, V_{max} can be readily derived from the rate of catalytic reaction measured at different concentrations of SDM. The relationship between removal rate and initial SDM concentration is shown in Figure 4.45. The linear plot between decolorization rate and SDM concentration depicts that, removal rate is increasing with increasing dye concentration. This can be explained as in lower dye concentrations, the microorganisms directs energy for growth instead of dye decolorization and the enzymes found it difficult to recognize dye molecules at lower dye concentration. This results in slower decolorization rate.

The double reciprocal plot of decolorization rate against SDM concentration yields a straight line (Figure 4.46) with an intercept of $1/V_{max}$ and a slope of K_m/V_m. Hence the values obtained from this study signifies that, the maximum decolorization rate V_m of 69.93 h^{-1} was reached when SDM concentration was greater than K_m i.e. 693.188 mg/L.

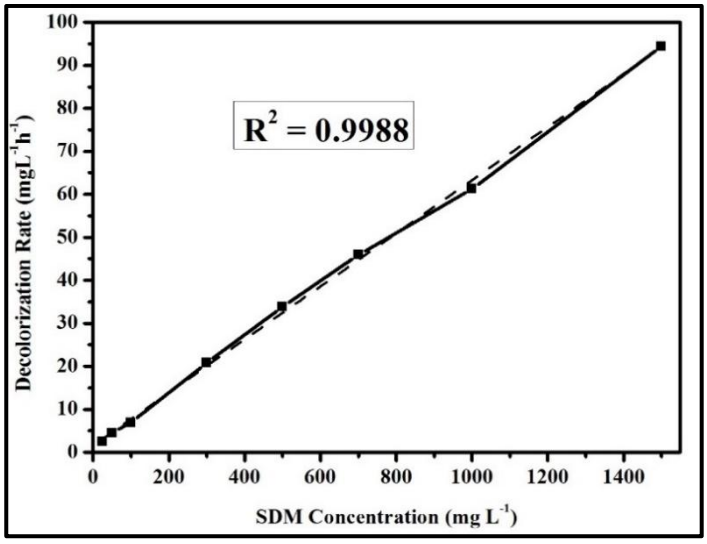


Figure 4.45: Effect of SDM initial concentration on SDM decolorization rate using "MA12C" consortium

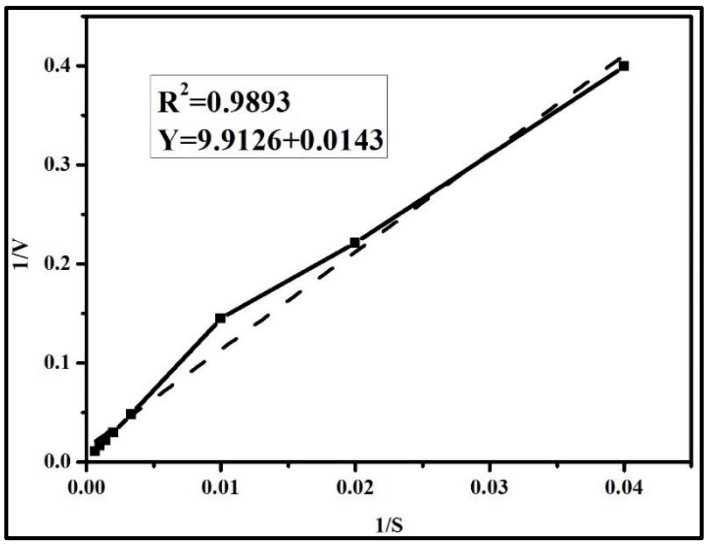


Figure 4.46: The double reciprocal plot of initial decolorization rate (mg L^{-1} h^{-1}) against SDM concentration (mg L^{-1}) to calculate V_{max} and K_m using "MA12C" consortium

4.2.7 Conclusion

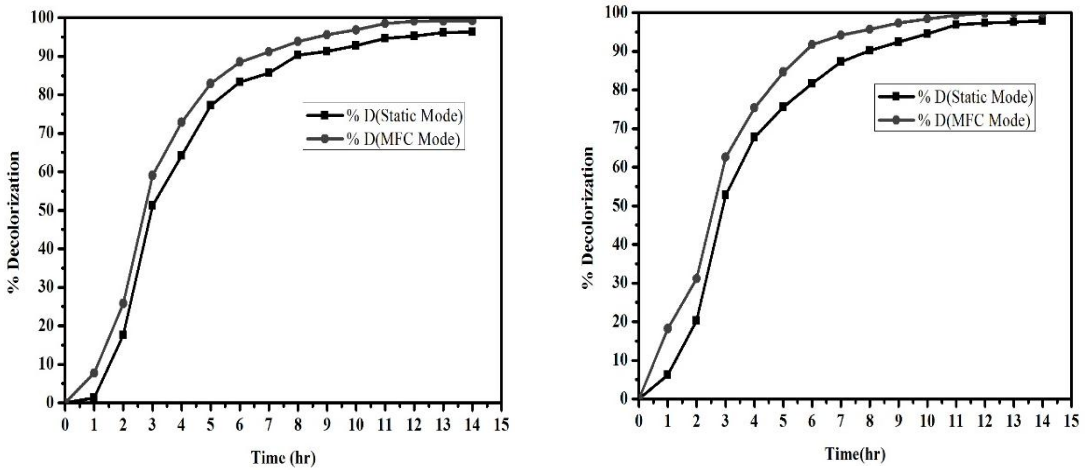
This work investigated the dye degradation efficiency of two novel bacterial consortium developed from the isolated bacterium from dye contaminated textile wastewater used in previous studies. Both the novel consortium were found to be more efficient in color removal as compared to the pure cultures. Consortium-1 designated as "M12C" comprises of two bacterial strains *Zobellella taiwanensis* AT 1-3 (M1C) and *Bacillus pumilus* (M2C) HKG212, and consortium-2 designated as "MA12C" comprising of three

bacterial strains i.e. *Bacillus pumillus* (M2C), *Zobella taiwensis* (M1C) and *Enterococcus durans* (M1A). Dye decolorization capacity of both the consortium was compared for the decolorization of an industrially important azo dye RG-19 in a very wide range of concentration (i.e. 100 mg/L to 1000 mg/L), where "MA12C" consortium was found to show slightly higher decolorization efficiency as compared to "M12C" consortium. A complete study has been carried out to evaluate the dye decolorization and degradation capacity of "M12C" consortium for the decolorization of an industrially important textile azo dye Reactive green-19. Under optimized condition the consortium was able to achieve 97% color removal at 100 mg/L dye concentration in 24h of incubation. The optimal decolorization conditions obtained for "M12C" through optimization using response surface methodology were as follows: temperature, 32.04°C; pH, 8.3 and Yeast Extract concentration, 1.16 g/100 mL. Taking into consideration, the industrial applicability of the developed consortium, "MA12C consortium was studied to check its degradation capacity for mixture of three structurally different azo dyes. Under optimized condition, "MA12C" consortium was able to remove 98% of SDM in 14h of incubation. The validation experiment results for both the consortia clearly confirmed the efficacy of Box–Behnken experimental design for RSM as an effective tool for mathematical modeling and factor analysis of decolorization process using a bacterial consortium. Both the consortia under study followed first order kinetics for the decolorization of dye molecules. It was found that decolorization of both RG-19 and SDM followed first order kinetics and depicted that decolorization rate increases with increase in initial dye concentration. The overall studies revealed that combined metabolic activity of different strains results in enhanced decolorization efficiency of the two developed consortia. The ability of the bacterial consortium "MA12C" comprising of three dominated bacterial strains isolated from dye contaminated wastewater to effectively decolorize SDM under saline condition indicated its potential application for textile effluents contaminated with azo dyes and other similar toxic compounds.

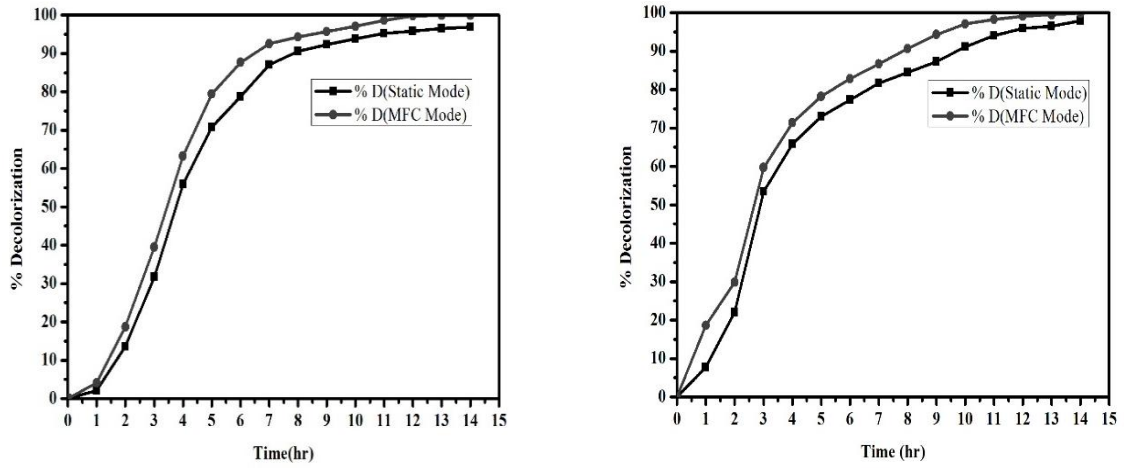
4.3 Complete degradation of Remazol navy blue in an integrated MFC-Aerobic two stage system and simultaneous bioelectricity generation using the developed consortium “MA12C”

4.3.1 Comparison of Decolorization of Different Textile Azo Dyes in Static and MFC Mode Using “MA12C” Consortium

Decolorization potential of the developed consortium “MA12C” for the decolorization of three model dyes (RG-19, RNB & RR-198) and Synthetic dye mixture (SDM) has been compared in traditional static and MFC mode. Medium containing 100 mg/L of dye was inoculated with “MA12C” consortium and the extent of decolorization was monitored in different time intervals. Figure 4.47 suggests that, under optimized condition, the consortium was found to be highly effective in decolorization of all the model azo dyes both in static and MFC mode. However, in MFC mode of operation, the dye decolorization process was found to be faster with an increased decolorization efficiency as compared to the traditional static incubation condition. In MFC mode, almost complete decolorization of all the model dyes has been achieved within 12hr of operation, whereas traditional static incubation condition maximum color removal of 97.89%, 96.22%, 96.9% and 97.93% was obtained for RNB, RG-19, RR-198 and SDM respectively after 14 h of incubation.



(a) **(b)**



(c) **(d)**

Figure 4.47: (a) Decolorization of RG-19 in Static and MFC mode; (b) Decolorization of RNB in Static and MFC mode; (c) Decolourization of RR-198 in Static and MFC mode; (d) Decolourization of SDM in Static and MFC mode

Similar findings were reported by Sun et al.(2009) for the decolorization of azo dye Active brilliant red X3 (ABRX3) in a single chambered air-cathode MFC using a mixture of aerobic and anaerobic sludge as inoculum [45]. They achieved complete decolorization of ARRX3 dye within 48h of MFC operation at an initial dye concentration of 300 mg/L, while only 80.1% color removal was obtained with traditional anaerobic reactor. Azo dye reduction under anaerobic condition occurs through direct electron transfer to azo dyes via bacterial enzymes produced during bacterial catabolism [253]. Presence of anode and aerated cathode in MFCs increases the metabolic rate of bacteria in MFC environment by providing sufficient anaerobic terminal electron acceptors [38,254]. This results in faster substrate utilization by bacteria to produce more electrons which leads to increased rate of azo dye reductive cleavage.

4.3.2 Effect of Dye Concentration on Removal Efficiency of "MA12C" Consortium in Static & MFC Mode

Effect of RNB concentration on decolorization efficiency of "MA12C" strain was found to follow same trend both in the static and MFC mode (Figure 4.48). However, a slightly higher decolorization of RNB has been achieved in MFC mode of operation. In MFC mode the consortium was found to be highly efficient in decolorizing RNB in a very wide concentration range (25 mg/L to 1000 mg/L). At low to moderate RNB concentrations

(25-100 mg/L), nearly complete decolorization of RNB was noticed and with 1000 mg/L dye concentration, more than 95% of decolorization has been achieved within 12h of MFC operation. The decolorization efficiency starts decreasing with increase in dye concentration. This can be attributed to the fact that, at higher concentrations the dye and its metabolites exert toxic effect on the microorganisms, which result in decreased metabolic rate and hence decolorization efficiency decreases [38,39].

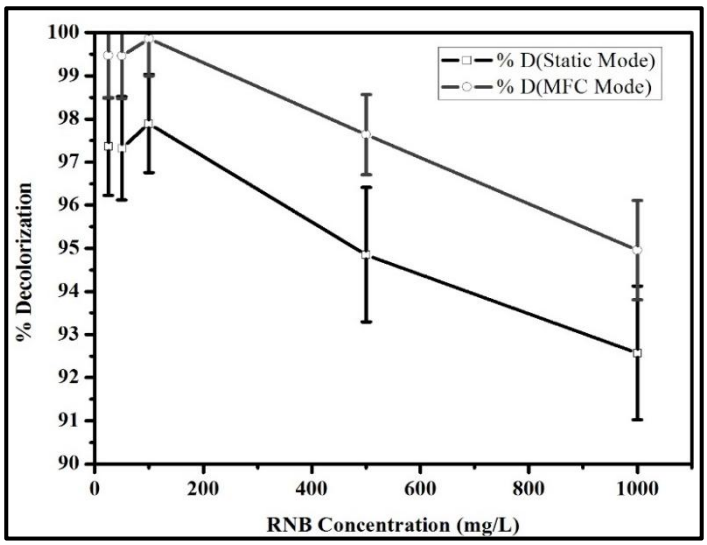


Figure 4.48: Effect of RNB concentration on removal efficiency of "MA12C"

4.3.3 Assessment of Simultaneous Power Production During RNB Decolorization in MFCs

The electrochemical performance of MFCs operated under different initial dye concentrations were evaluated from the polarization curves plotted between power density vs current density at different resistances (Figure 4.49). RNB concentration in the anode compartment up to concentration of 100 mg/L was found to have no apparent effect on the maximum power production efficiency of the MFC as compared to the control MFC operated with the absence of RNB. The maximum power density of 52.5 mW/m^2 was produced from the MFC system with 1000 Ω resistor which was found to decrease to 45.9 mW/m^2 when the RNB concentration was increased to 500 mg/L. As the concentration of RNB increases in the anode compartment, significant portion of electrons released from the microbial metabolism gets diverted to reduce the dye

chromophore. Hence presence of alternative electron acceptor in abundance affects the electrochemical performance of MFC which results in decreased power density.

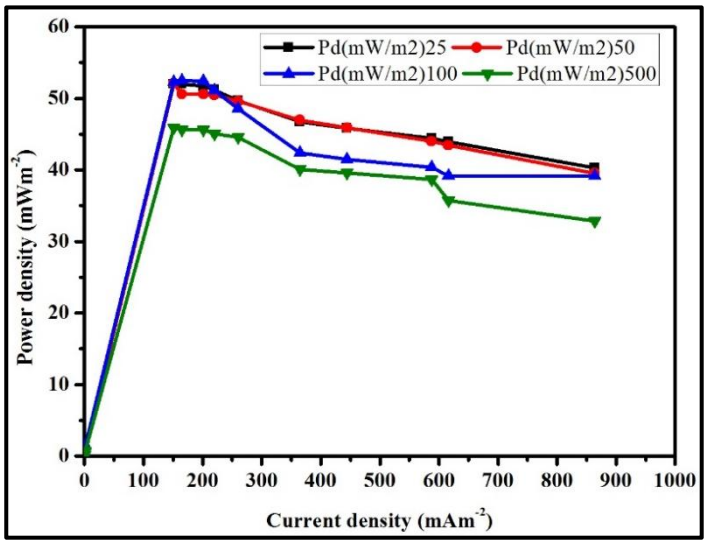


Figure 4.49: Power density- Current density plots during during RNB decolorization in MFC system at different RNB initial concentrations using "MA12C"

4.3.4 Study of Decolorization Kinetics of RNB in MFC System

Figure 4.50 depicts the decolorization kinetics of RNB dye in MFC anode with 100 mg/L initial dye concentration. The correlation coefficient R^2 = 0.9719 for the plot $\ln(C_0/C_t)$ vs time (t) approximates that, RNB decolorization kinetics in MFC anode well fitted to first order decay model as described in equation (3.5).

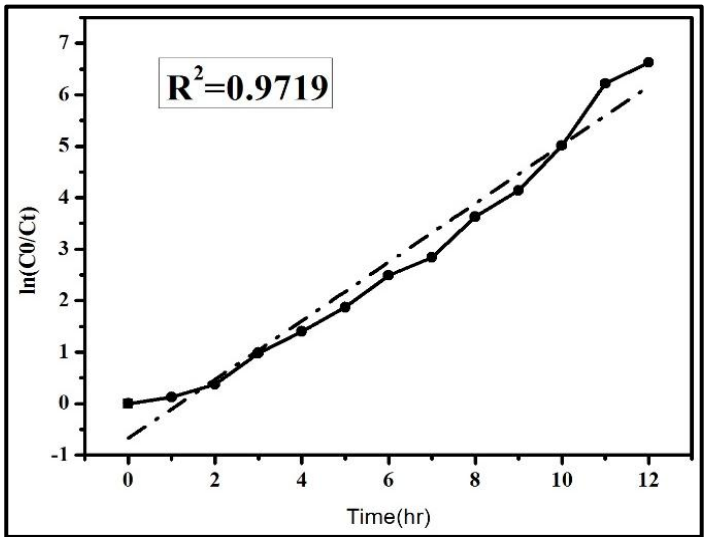


Figure 4.50: First order logarithmic decay model of RNB removal in MFC anode using "MA12C"

Table 4.12 suggests that, the first order decolorization constants (k) for the decolorization of RNB decreased with increase in RNB concentration. This may be due to the toxic effect of dye and its degradation metabolites which decreases the decolorization efficiency of anodic microbial population.

Table 4.12 also compares the decolorization rate constants for decolorization of RNB in static and MFC mode. For each initial concentration of RNB, decolorization rate constant in MFC mode was found to be considerably higher in comparison to that of static mode. This could be attributed to the fact that, unlike anaerobic digestion in batch mode, MFCs use oxygen as the final electron acceptor in cathodic chamber which enhances the microbial metabolism. Due to increased microbial metabolic rate, more reducing equivalents are available for reduction of azo bond which leads to faster decolorization rate [34,38].

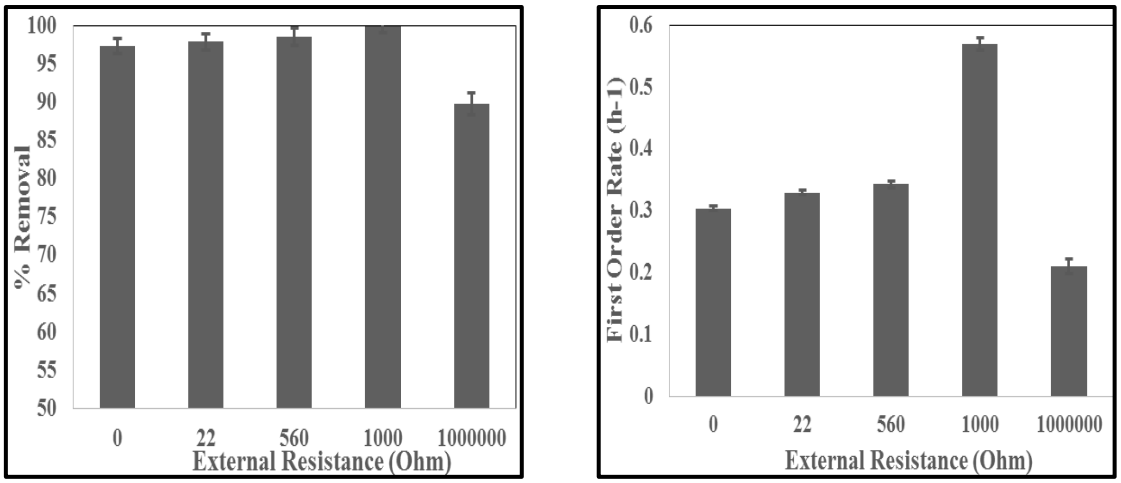
Table 4.12: Effect of dye conc. on first order rate constant

RNB Concentration (mg/L)	**First order rate constant (h^{-1}) (k)**	
	Static Mode	**MFC Mode**
25	0.4468	0.6713
50	0.4582	0.5932
100	0.3041	0.5697
500	0.2488	0.3338
1000	0.2143	0.2675

4.3.5 Effect of External Resistance on Anodic Decolorization of RNB and Kinetics of RNB Removal

MFCs external resistance have been reported to be an important parameter which significantly affects the reductive degradation of dye molecule in anodic compartment [45,137]. Optimum external resistance affects the external electron transfer efficiency of anodic microorganisms which eventually affects the degradation of azo bond. In current study, MFCs were operated with four different external resistances between anode and cathode to explore the effect of external resistance on decolorization of RNB (100 mg/L).

It was observed that, with increase in external resistance, the removal efficiency and reductive decolorization rate constants of RNB were found to increase and are significantly higher than the open circuit control. This may be due to efficient transfer of electrons to the azo dye moiety in electronically active environment of MFC anode with higher external resistance. Complete decolorization was achieved with 1000 Ω resistor and with further increase in external resistance to 1MΩ, the percentage decolorization of RNB decreased to 89% (Figure 4.51a). Similar trend has been observed for decolorization rate constant for decolorization of RNB in MFC anode (Figure 4.51b). With increasing external resistance, more reducing equivalents would be available for the reduction of dye chromophore which results in increasing percentage color removal. However, much higher external resistance like 1MΩ creates unfavorable environment for exoelectrogens which leads to decrease in decolorization efficiency [137]. Similar findings were reported by some researchers where electrochemically active environment of MFCs favored rapid decolorization of different azo dyes [137,255].



(a) **(b)**

Figure 4.51: (a) Effect of MFC external resistance on RNB %Removal (b) Effect of MFC external resistance on first order rate constant (h^{-1}) of RNB decolorization using "MA12C"

4.3.6 Effect of External Resistance on Power Production in MFCs Coupled to Degradation of Different Azo Dyes

The maximum power density achievable from MFCs operating under various external resistances followed the similar trend to the decolorization kinetic constants and percentage removal for decolorization of RNB dye under similar condition in MFC

system. Three structurally different azo dyes (100 mg/L) i.e. RG-19, RR-198 and RNB were utilized in MFCs and the maximum power produced during decolorization of these dyes under different external load has been noted. Figure 4.52 illustrates the effect of external resistance on the maximum power production capacity of MFCs with simultaneous decolorization of different textile azo dyes. The results indicate that, for all the MFCs treating different azo dyes, maximum power density (P_{max}) has been achieved with 1000 Ω resistor. Maximum power density of 52.5 mW m^{-2}, 51.85 mW m^{-2} and 51.98 mW m^{-2} has been obtained during decolorization of RNB, RG-19 and RR-198 respectively in anodic compartments of MFCs. The power density was found to decrease with increase or decrease of external resistance beyond the optimum value. This implies the maximum power density obtained from the MFCs during decolorization of different azo dyes was found to be significantly affected by the external resistance which is in agreement with the findings of Mennicucci et al. (2005) and Eustace et al. (2013) [137,172].

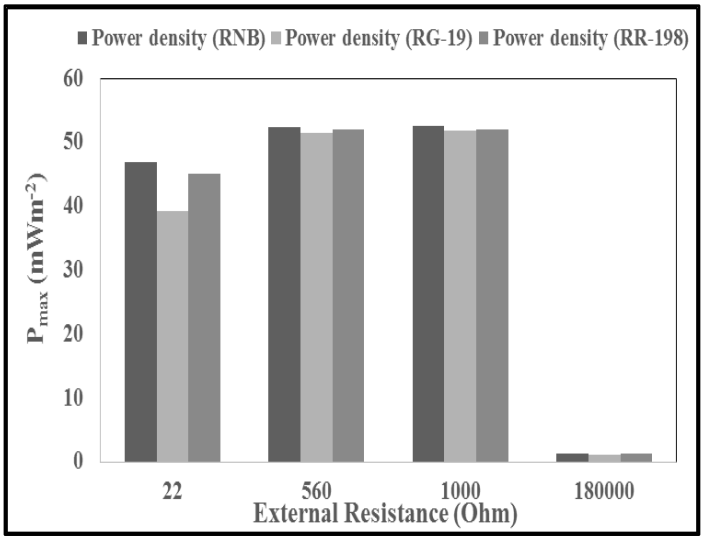


Figure 4.52: Effect of external resistance on maximum power density obtainable from MFC during degradation of 100 mg/L concentration of RG-19, RNB and RR-198 using "MA12C" consortium

4.3.7 Comparison of Azo Dye Decolorization and Simultaneous Electricity Production Capacity of the Developed MFC System with Similar Studies in Literature

Table 4.13 relates our findings with that of different MFC configurations and different inocula for decolorization of different azo dyes reported in other studies. It is evident from the comparison that, MFC configuration and electrode material plays a major role in increasing MFC performance for decolorization of different azo dyes and simultaneous electricity production. Sun et al. (2009) and Waheed et al. (2015) reports the maximum power density as 234 mW/m^2 and 455.7 mW/m^2 respectively which was considerably higher as compared to other studies due to the use of Pt coated cathode. Cheng et al.(2006) suggested that the power density obtained using Pt catalyst on cathode surface were four times more than those obtained using plane carbon electrodes [256]. Li et al. (2010) used two types of MFCs with varying cathode materials and observed a considerable increase in power density with Graphite granular cathode as compared to carbon felt (cathode material). They obtained maximum power density of 256.33 mW/m^2 with granular graphite cathode which is much higher than 59.16 mW/m^2 as obtained with carbon felt cathode. In our studies we have used carbon cloth as the electrode material for anode and cathode. The maximum power density obtained was 52.5 mW/m^2 which is higher than that obtained by Eustace et al. (2012) (37 mW/m^2) using the same cathode material which may be attributed to varying metabolic activity of the organisms. It is further confirmed when color removal efficiency of different MFC systems is compared with our developed MFC using MA12C consortium shows faster decolorization rate with 100% color removal within 12 h of operation.

Table 4.13 Comparision of colour removal using MFC

Inoculum type	MFC type	Target azo dye with initial concentration	% Color removal	Maximum Power density obtained	Reference
Shewanella oneidensis	Dual chambered MFC with carbon cloth as anode and cathode	Acid orange7 (70mg/L)	90% within 30hr	37 mW/m^2	[38]

Mixture of aerobic and anaerobic sludge	Air-cathode single chambered MFC with porous carbon paper as anode and carbon paper with Pt catalyst layer as cathode	Active brilliant red X-3B (300mg/L)	100% within 48hr	234 mW/m^2	[45]
Anaerobic sludge	Dual chambered MFC with Graphite felt as anode and Pt coated Graphite cloth as cathode	Direct red 80 (200 mg/L)	85% within 48hr	455.7 mW/m^2	[39]
Anaerobic sludge	Dual chambered MFC with Carbon felt as anode and Graphite granule as cathode	Congo red (100 mg/L)	92.7% within 14.8hr	256.33 mW/m^2	[163]
Anaerobic sludge	Dual chambered MFC with Carbon felt as anode and cathode	Congo red (100 mg/L)	–	59.16 mW/m^2	[163]
Defined consortium	Dual chambered MFC with Carbon cloth as Anode and cathode	Remazol navy blue (100mg/L)	100% within 12hr	52.5 mW/m^2	**Current study**

4.3.8 Analysis of Degradation Products of RNB Degradation in Integrated MFC-Aerobic Sequential Process

- **UV-VIS Spectral Analysis**

If the dye removal attributed to biodegradation, either the major visible light absorbance peak will completely disappear, or a new peak will appear [257]. UV-VIS spectra of the effluent collected from end of each process of the two-stage system for degradation of RNB has been recorded (Figure 4.53). Complete disappearance of the absorption peak at

597 nm in the visible region suggests the breakdown of the chromophore at the end of anaerobic treatment using the developed consortium [253,257]. The decrease in peak at 340 nm and 259 nm indicates the destruction of aromatic rings in successive aerobic treatment process [163].

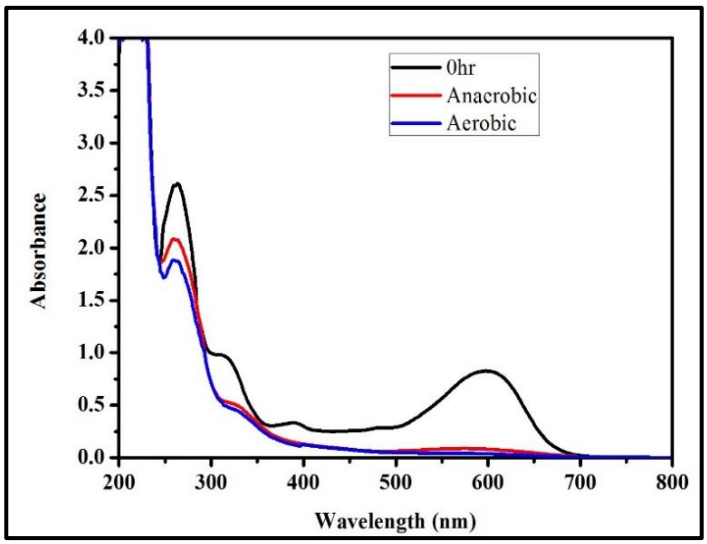
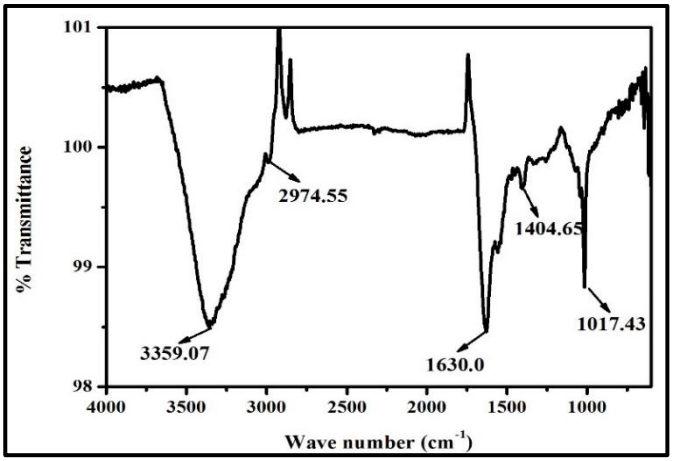


Figure 4.53: UV-Vis spectra of effluent from MFC system and aerobic chamber for decolorization of RNB using "MA12C" consortium

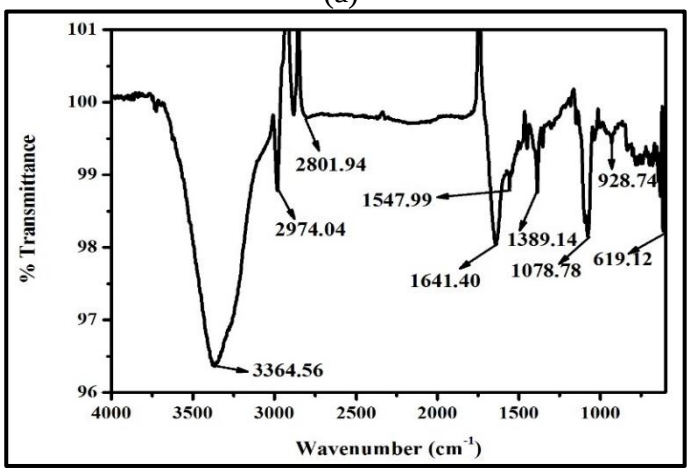
- **FTIR Analysis**

FTIR analysis of the dye degradation metabolites obtained after each stage treatment of RNB dye in the MFC-aerobic two stage system was carried out to provide an additional insight to the biotransformation of the dye molecule. FTIR spectra obtained from the control dye RNB (Figure 4.54a) showed specific peaks at 3359cm^{-1} for –C-H stretching of alcoholic or phenolic compound, 2974cm^{-1} for alkanes, 1775 cm^{-1} for reactive carbonyl group, 1630cm^{-1} for –N=N- stretching of azo bonds, 1404cm^{-1} for –OH deformation of alcohols, 1017cm^{-1} for aliphatic phosphate group (P-O-C stretch).

Metabolites obtained after first stage anaerobic treatment of RNB dye in MFC by the developed consortium showed a major peak at 3364.56 cm^{-1} suggesting formation of amines after anaerobic degradation of the dye by the developed consortium (Figure 4.54b). Peak at 1641.40 cm^{-1} signifies presence of N-H bend of amides, peak at 1547.99 cm^{-1} for N-H bend of secondary amines and peak at 1078.78 cm^{-1} signifies C-N stretch of aliphatic amines.



(a)



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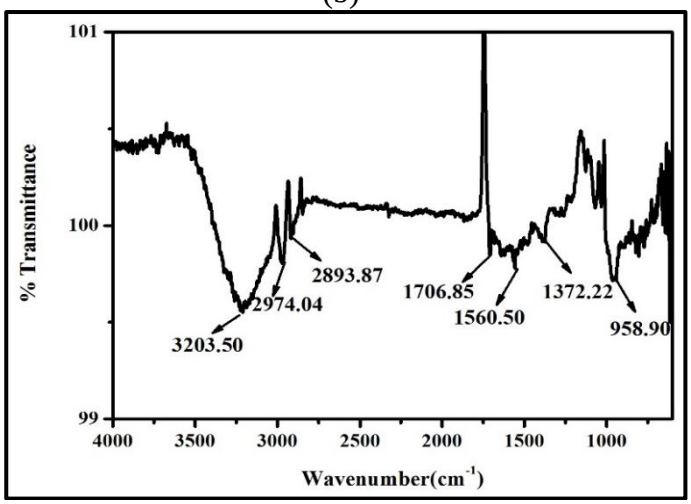


(c)

Figure 4.54: (a) FTIR spectra of control RNB before treatment; (b) FTIR spectra of RNB degradation metabolites after anaerobic treatment in MFC stage; (c) FTIR spectra of RNB degradation metabolites after aerobic treatment in aerobic chamber

Peak at 2801.94 cm^{-1} and 2974.04 cm^{-1} for O-H stretch of carboxylic acids and at 928.74 cm^{-1} for O-H bend of carboxylic acids signifies formation of different carboxylic acid

isomers. Peak at 1389.14 cm^{-1} signifies N=O bend of Nitro group and peak at 619.12 cm^{-1} signifies C-H bend for alkynes. Moreover, the absence of peak at 1630 cm^{-1} for N=N indicates the cleavage of azo bond [25,201].

Analysis of FTIR results of the metabolites obtained from the effluent from aerobic chamber after the two stage treatment does not show any significant peaks in (3300 cm^{-1}–3500 cm^{-1}) region suggesting absence of amines in the produced metabolites (Figure 4.54c). Peaks at 2893.87 cm^{-1} and 2974.04 cm^{-1} for C-H stretch of alkanes, 1372.22 cm^{-1} for C-H rock of alkanes suggests degradation of complex dye structure into simple metabolic products. Peak at 1554.61 cm^{-1} suggests C=C for aromatic compounds, 3203.50 cm^{-1} for O-H stretch of alcohols or phenolic compounds, 1712.74 cm^{-1} for C=O stretch of carboxylic acids and peak at 958.90 cm^{-1} signifies O—H bond of carboxylic acid group.

A considerable difference in the FTIR spectrum of control dye and the metabolites obtained from both anaerobic and aerobic stage treatment confirmed the biodegradation of the dye into different metabolites. Amines produced during the anaerobic treatment of the dye in MFC system were found to be eliminated in the successive aerobic treatment stage using the same developed bacterial consortium.

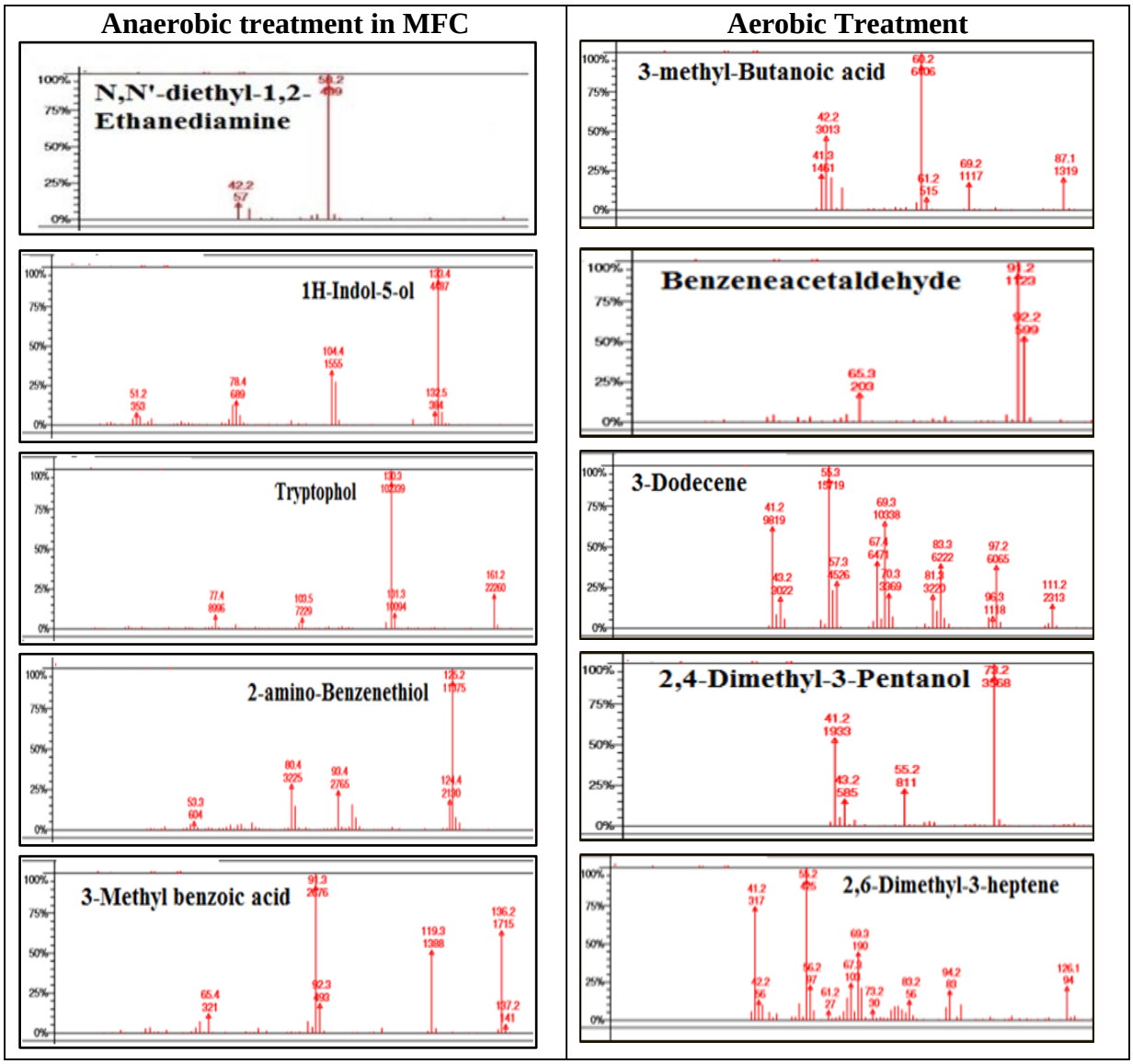
- **GC-MS Analysis**

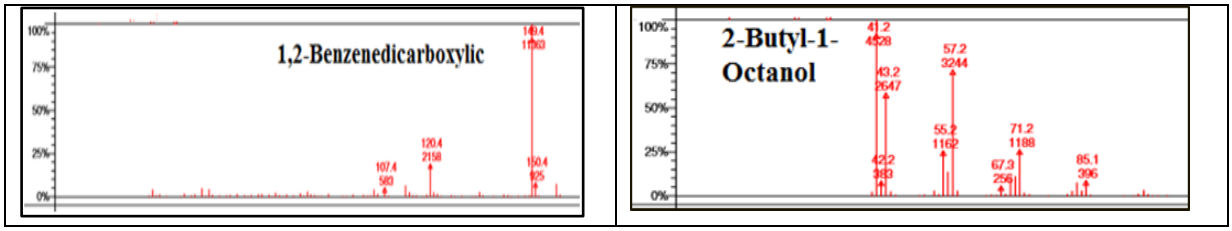
GC-MS analysis of the degradation metabolites obtained from each stage were carried out and compared to identify the degradation products formed after the anaerobic and subsequent aerobic treatment of RNB (Table 4.14). Mass spectrum analysis and NIST library, suggests proposed metabolites formed after anaerobic treatment of RNB dye in anodic chamber of MFC to be amines, unsaturated carboxylic acids and organic sulfides. The amine isomers might be (N, N'-diethyl-1,2-Ethanediamine) at a retention time of 6.112 min with mass peak of 116, (1H-Indol-5-ol) at a retention time of 15.776 min mass peak at 133 and (Tryptophol) at retention time of 17.8 min with mass peak 161. These compounds have toxic effect on human and animals causing skin and respiratory infection. Tryptophol was reported to show genotoxicity in-vitro [258].The organic sulfide detected is supposed to be (2-amino-Benzenethiol) at a retention time of 11.955 min with mass peak of 125. The unsaturated carboxylic acid isomers might be (3-Methyl benzoic acid) at retention time of 11.069min and (1,2-Benzenedicarboxylic acid) at

retention time of 18.593 min. These compounds also sometimes found to show negative effect on human and animals causing various health hazards [259].

GC-MS analysis of the metabolites obtained at the end of aerobic second stage treatment suggests the oxidative degradation of different aromatic amines into different simpler metabolites.

Table 4.14: GC-MS peaks of RNB degradation metabolites obtained after treatment in MFC stage and aerobic stage





GC-MS analysis and NIST library suggest that, the formation of (3-methly –Butanoic acid) at retention time of 4.199 min, (Benzeneacetaldehyde) at retention time of 7.737 min, (2, 4-Dimethyl-3-Pentanol) at retention time of 3.244 min, (2,6-Dimethyl-3-heptene) at retention time of 6.629 min, (2-Butyl-1-Octanol) at retention time of 9.912 min and (3-Dodecene) at retention time of 17.620 min. This result implies the formation of different simpler straight chain compounds and breakdown of complex dye and its toxic intermediates.

Hence it can be assumed that treatment of azo dye RNB in MFC-aerobic sequential system results in complete mineralization of the dye molecule into simpler metabolites which have less toxic effect than that of the parent dye molecule. Toxic effect of the parent dye and its degradation metabolites were analyzed using phytotoxicity study.

- **Phytotoxicity analysis**

Toxic nature of azo dyes and their degradation products is of great concern as it causes serious environmental hazard, affecting plants and animals when released into the water stream without proper treatment. Biodegradation of azo dye in anaerobic environment often produces amines, which need further treatment due to its obvious toxic effect on environment [260,261]. Plant bioassay using *P. mungo* has been used to study the toxicity level of RNB dye and its degradation metabolites (Table 4.15). 60% inhibition in germination was noticed with 300 mg/L dye solution unlike nearly complete germination and significant plant growth observed for plants grown in same concentration of dye degradation metabolite solutions. In addition to this, percentage germination and plant growth was found to be lower in seeds germinated with metabolites obtained from anaerobic treatment of dye in MFC system as compared to that of metabolites obtained from aerobic treatment. This may contribute to the fact that, aromatic amines were formed during anaerobic degradation of azo bond making the effluent toxic for plant growth and germination which is eliminated in subsequent aerobic treatment process. Sequential

anaerobic- aerobic treatment helped in complete mineralization of azo dyes into non-toxic intermediates which resulted in lowering toxic effect of the effluent on plant growth.

Table 4.15: Phytotoxicity study of RNB and its degradation metabolites formed after anaerobic treatment in MFC chamber and subsequent aerobic treatment on *Phaseolus mungo*

Parameters studied	**Distilled water**	**RNB dye solution**	**Metabolites from MFC anaerobic treatment**	**Metabolites from Final aerobic treatment**
Germination (%)	100	40	98	100
Shoot length (cm)	12.82 ± 0.51	2.61 ± 0.39*	9.49 ± 0.40**	11.11 ± 0.72***
Root length (cm)	3.35 ± 0.38	0.81 ± 0.05*	2.42 ± 0.22**	3.24 ± 0.36***

Data was analyzed by one-way analysis of variance (ANOVA) with Turkey-Kramer multiple comparison test. The values are mean values of three experiments. Seeds germinated in RNB dye solution, metabolites from MFC anaerobic treatment and aerobic treatment stage are significantly different from the seeds germinated in distilled water at p* < 0.001, p**< 0.001, p*** < 0.001.

4.3.9 Conclusion

This study demonstrates the successful utilization of the developed consortium "MA12C" for decolorization of different textile azo dyes and simultaneous production of electrical energy using MFC system. As mentioned earlier, "MA12C" consortium comprises of three most dominant bacterial strains isolated from dye contaminated wastewater showing highest dye decolorization capacity, the outcome of the present investigation also indicates the possibility of utilizing bacterial species isolated from dye contaminated sites in integrated MFC-aerobic system for complete mineralization of colored effluent. Dye decolorization performance of "MA12C" consortium in anodic chamber of MFC was found to be higher than traditional anaerobic condition. Complete decolorization of RNB dye has been achieved within 12h of MFC operation with initial dye concentration of 25-100 mg/L. At higher dye concentration (1000 mg/L) the decolorization efficiency slightly reduced to 95% due to toxic effect of dye and its degradation metabolites.

MFC external resistance was found to be an important parameter influencing the overall efficiency of MFC system and could be utilized as a potential tool to achieve enhanced

azo dye reductive decolorization kinetics. External resistance of 1000 Ω was found to be optimum for achieving maximum dye removal.

Studies of dye degradation metabolites from each stage of the integrated MFC-aerobic sequential process through different analytical techniques like UV-Vis spectrophotometer, FTIR and GCMS analysis demonstrates that, partial degradation and decolorization of RNB dye in MFC chamber (first stage anaerobic treatment) resulted in formation of amines and some toxic intermediates. However, effluent released from second stage aerobic treatment process achieved removal of toxic amines under microaerophilic conditions. The results obtained for analysis of dye degradation products through different analytical techniques has been supported by the phytotoxicity study. The study confirms the less toxic nature of effluent released from second stage aerobic treatment as compared to the control dye and metabolites obtained from first stage of anaerobic treatment of RNB dye using our developed consortium "MA12C".

Therefore, it can be concluded from this study that, our developed consortium comprising of three bacterial strains *Zobellella taiwanensis* AT 1-3, *Bacillus pumilus* HKG212 and *Enterococcus durans strain* GM13 could potentially be employed in a two stage MFC-aerobic biological treatment process to achieve higher decolorization rate as well as complete mineralization of the dye molecule into nontoxic intermediates.

Chapter 5

Conclusions and Future Prospective

5.1 Conclusions

The main outcome of this work is the isolation of novel bacterial strains from textile wastewater that can effectively degrade three different textile azo dyes (Reactive green-19 (RG-19), Remazol navy blue (RNB), Reactive Red-198 (RR-198)). Isolated bacteria species showed high dye degradation potential as compared to pure strain when formed consortiums such as M12C and MA12C. Most importantly MA12C could effectively decolorize RG-19 dye than M12C. M12C comprised of *Bacillus pumilus* HKG212 and *Zobellella taiwanensis* AT 1-3 whereas MA12C included *Bacillus pumilus* HKG212, *Zobellella taiwanensis* AT 1-3 and *Enterococcus durans* GM13. Individual and synergistic effect of different process parameters affecting the decolorization efficiency of the two developed consortia were investigated and optimized using Box-Behnken design. Dye decolorization kinetic parameters for the decolorization of different dyes using the two consortium were estimated. The beauty of the work lies on the development of a Microbial fuel cell (MFC) system using MA12C consortium to speed up the anaerobic degradation of azo dyes and simultaneous production of biogenic electricity. An integrated MFC-aerobic sequential system has been developed using the consortium MA12C as the inoculum to achieve complete mineralization of the azo dye Remazol Navy Blue. Different analytical techniques such as UV-Vis spectrophotometer, FTIR and GC-MS has been used to identify the dye degradation metabolites formed during the degradation of dye in each stage of the sequential treatment system. Toxicity of the effluent after degradation of RNB has been evaluated using phytotoxicity analysis. Some important findings have been reported below:

- The three most dominant bacterial strains showing maximum decolorization efficiency were identified using 16s rRNA analysis as *Enterococcus durans* GM13, *Zobellella taiwanensis* AT1-3 and *Bacillus pumilus* HKG212.

- Isolated strains were found to be capable of decolorizing all the three model dyes (i.e. RG-19, RNB and RR-198) in a wide concentration range (100 mg/L – 1000 mg/L) and achieved 80-95% decolorization within 24h of incubation.
- Ambient temperature, neutral to slightly alkaline pH, anaerobic/static incubation condition was found to be suitable for achieving maximum decolorization potential for all the three isolates.
- Two defined consortia were developed using the isolated strains, among those, MA12C was found to be more efficient than M12C for decolorization of RG-19.
    - Using response surface methodology and Box-Behnken design, the optimum values of process parameters obtained for achieving maximum decolorization efficiency of M12C consortium was temperature (32.04°C), pH (8.35), Yeast extract concentration (1.16 g/100mL) and for MA12C consortium the optimum conditions are temperature (29.9°C), pH (8.29), Yeast extract concentration (1.88 g/100mL).
    - Under the optimum conditions M12C achieved 97% decolourization of 100 mg/L RG-19 and 97.95% decolorization of SDM has been attained using MA12C consortium.
- Feasibilty of using MA12C consortium has been investigated in a two stage integrated MFC-aerobic system for complete mineralization of azo dye RNB.
    - A higher decolorization efficiency of the consortium was observed in MFC system for decolorization of different textile azo dyes as compared to the traditional anaerobic treatment method. Nearly complete decolorization was noticed from low to moderate RNB concentration and with 1000 mg/L dye concentration, more than 95% of decolorization has been achieved within 12h of MFC operation.
    - Maximum power production capacity got slightly affected with increasing dye concentration.
    - MFC external resistance was found to be an important parameter affecting the MFC performance.

- UV-Vis, FTIR and GC-MS analysis of RNB degradation products showed the presence of some toxic intermediates such as amines, unsaturated carboxylic acids and organic sulfides in the effluent collected MFC system. However, detection of some straight chain organic compounds and absence of these toxic intermediates in the effluent released from subsequent aerobic treatment stage suggests the complete mineralization of RNB dye.
- Phytotoxicity study using *P. mungo* confirms the less toxic nature of effluent released from each stage of the integrated MFC-aerobic system as compared to the parent RNB dye.

The above studies suggest that, organisms isolated from dye contaminated wastewater can potentially be utilized for treatment of different azo dyes and simultaneous production of bioelectricity. Our developed consortium comprising of three bacterial strains *Zobellella taiwanensis* AT 1-3, *Bacillus pumilus* HKG212 and *Enterococcus durans strain* GM13 found to be effective in both anaerobic and anaerobic treatment stage of the integrated MFC-aerobic system for treatment of azo dye RNB. Hence, these organisms could potentially be employed in a two stage MFC-aerobic biological treatment process to achieve higher decolorization rate as well as complete mineralization of the dye molecule into nontoxic intermediates.

5.2 Future Prospective

This study suggests the feasibility of using indigenous microorganisms present in dye contaminated wastewater in electrochemically active environment like MFCs to achieve an enhanced rate of decolorization and simultaneous production of bioelectricity. Decolorization potential in MFC was higher than that obtained from traditional anaerobic treatment method. However, the electrical power generated was quite low. Hence some future studies should be carried out to increase the MFC performance for degradation of azo dyes and concomitant production of electrical energy. Hence, the final outcome of this work highlights several areas for future studies which will enhance the efficiency of the current technology to treat colored industry wastewater.

- Improvement of MFC configuration and choice of MFC elements.
- Development of biocathode to reduce the cost.
- Use of enzymes as catalyst in cathodic chamber.
- Use of molecular biology approach for producing recombinant microorganisms that are having higher efficiency of extracellular electron transfer.
- A better understanding of the factors affecting the behavior of microorganisms in electrochemically active environment is necessary to enhance the efficiency of the process.
- Better understanding of the enzymes involved and the metabolic pathways followed by the organisms during azo dye reduction and simultaneous electricity production is required for achieving enhanced bioremediation efficiency.

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Appendix-I

Table 1: Sequence Producing Significant Alignments (M1A)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
KC213478.1	Enterococcus durans strain GM18	2481	2481	100%	0.0	99%
NR_114015.1	Enterococcus thailandicus strain NBRC 101867	2481	2481	100%	0.0	99%
JN409464.1	Enterococcus thailandicus strain SP15	2481	2481	100%	0.0	99%
GU125447.1	Enterococcus thailandicus strain IMAU80025	2481	2481	100%	0.0	99%
GU125446.1	Enterococcus thailandicus strain IMAU80024	2481	2481	100%	0.0	99%
DQ411817.1	Enterococcus sanguinicola strain ss1743	2481	2481	100%	0.0	99%
AB511021.1	Enterococcus durans, strain: C102901	2481	2481	100%	0.0	99%
FJ378705.1	Enterococcus sanguinicola strain HN-S8	2481	2481	100%	0.0	99%
NR_044160.1	Enterococcus thailandicus strain FP48-3	2481	2481	100%	0.0	99%
KC213474.1	Enterococcus durans strain GM13	2477	2477	100%	0.0	99%
FJ607288.1	Enterococcus durans strain KLDS6.0632	2470	2470	100%	0.0	99%
FJ607266.1	Enterococcus durans strain KLDS6.0620	2470	2470	100%	0.0	99%
FJ607269.1	Enterococcus durans strain KLDS6.0622	2470	2470	100%	0.0	99%
FJ607249.1	Enterococcus durans strain KLDS6.0613	2470	2470	100%	0.0	99%
FJ607262.1	Enterococcus durans strain KLDS6.0617	2470	2470	100%	0.0	99%

Table 2: Sequence Producing Significant Alignments (M1C)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
NR_043630.1	Zobellella taiwanensis strain ZT1	2385	2385	100%	0.0	99%
FJ999669.1	Zobellella taiwanensis strain AT 1-3	2377	2377	100%	0.0	99%
KJ197182.1	Zobellella taiwanensis strain F4	2374	2374	100%	0.0	99%

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
FJ999668.1	Zobellella taiwanensis strain AT 1-2	2370	2370	100%	0.0	99%
FJ999667.1	Zobellella taiwanensis strain AT 1-1	2355	2355	100%	0.0	99%
GU980766.1	Oceanimonas smirnovii strain SS2	2224	2224	100%	0.0	98%
KF147911.1	Oceanimonas denitrificans strain KMER1	2178	2178	99%	0.0	99%
DQ097665.1	Oceanimonas denitrificans strain F13-1	2178	2178	99%	0.0	99%
NR_043629.1	Zobellella denitrificans strain ZD1	2154	2154	99%	0.0	99%
NR_114185.1	Oceanimonas doudoroffii strain NBRC 103032	2154	2154	100%	0.0	95%
JQ068797.1	Oceanimonas doudoroffii strain SDT1S29	2019	2019	99%	0.0	95%
NR_109063.1	Oceanisphaera sediminis strain TW92	2012	2012	99%	0.0	95%
NR_043622.1	Oceanisphaera donghaensis strain BL1	2008	2008	100%	0.0	95%
NR_109099.1	Oceanisphaera ostreae strain T-w6	2004	2004	100%	0.0	95%
JQ045823.1	Oceanimonas doudoroffii strain SDT1S4	1977	1977	100%	0.0	95%

Table 3: Sequence Producing Significant Alignments (M2C)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
KJ741252.1	Bacillus pumilus strain HKG212	2459	2459	100%	0.0	99%
KJ672333.1	Bacillus safensis strain MUGA148	2459	2459	100%	0.0	99%
KJ672302.1	Bacillus safensis strain MUGA101	2459	2459	100%	0.0	99%
KJ660959.1	Bacillus pumilus strain B1	2459	2459	100%	0.0	99%
KJ572281.1	Bacillus pumilus strain S-7	2459	2459	100%	0.0	99%
KC851831.1	Bacillus safensis strain S1-F1	2459	2459	100%	0.0	99%

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
KJ417904.1	Bacillus pumilus strain AK2	2459	2459	100%	0.0	99%
KJ210650.1	Bacillus safensis strain WB-260	2459	2459	100%	0.0	99%
KJ210642.1	Bacillus safensis strain WM-249	2459	2459	100%	0.0	99%
KJ210640.1	Bacillus safensis strain WM-261	2459	2459	100%	0.0	99%
KF475865.1	Bacillus pumilus strain IHB B 6571	2459	2459	100%	0.0	99%
KF465837.1	Bacillus pumilus strain P27	2459	2459	100%	0.0	99%
KF017359.1	Bacillus safensis strain w121	2459	2459	100%	0.0	99%
KF010796.1	Bacillus safensis strain B24V	2459	2459	100%	0.0	99%
JX139736.1	Bacillus safensis strain BA64	2459	2459	100%	0.0	99%

Table 4: Distance Matrix of M1A

		1	2	3	4	5	6	7	8	9	10	11
M1A	1		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
KC213478.1	2	0.001		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
NR_114015.1	3	0.001	0.000		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
JN409464.1	4	0.001	0.000	0.000		0.000	0.000	0.000	0.000	0.000	0.000	0.000
GU125447.1	5	0.001	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.000	0.000
GU125446.1	6	0.001	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.000
DQ411817.1	7	0.001	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.000
AB511021.1	8	0.001	0.000	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.000
FJ378705.1	9	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000		0.000	0.000
NR_044160.1	10	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		0.000
KC213474.1	11	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	

Table 5: Distance Matrix of M1C

M1C	1		0.000	0.000	0.001	0.000	0.000	0.004	0.004	0.004	0.005	0.005
NR_043630.1	2	0.000		0.000	0.001	0.000	0.000	0.004	0.004	0.004	0.005	0.005
FJ999669.1	3	0.000	0.000		0.001	0.000	0.000	0.004	0.004	0.004	0.005	0.005
KJ197182.1	4	0.002	0.002	0.002		0.001	0.001	0.004	0.004	0.004	0.005	0.005
FJ999668.1	5	0.000	0.000	0.000	0.002		0.000	0.004	0.004	0.004	0.005	0.005
FJ999667.1	6	0.000	0.000	0.000	0.002	0.000		0.004	0.004	0.004	0.005	0.005
GU980766.1	7	0.018	0.018	0.018	0.019	0.018	0.018		0.005	0.005	0.005	0.003
KF147911.1	8	0.022	0.022	0.022	0.024	0.022	0.022	0.033		0.002	0.003	0.005
DQ097665.1	9	0.025	0.025	0.025	0.026	0.025	0.025	0.033	0.006		0.002	0.005
NR_043629.1	10	0.027	0.027	0.027	0.029	0.027	0.027	0.034	0.013	0.007		0.005
NR_114185.1	11	0.030	0.030	0.030	0.031	0.030	0.030	0.017	0.038	0.034	0.035	

Table 6: Distance Matrix of M2C

M2C	1		0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002
KJ741252.1	2	0.005		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
KJ672333.1	3	0.005	0.000		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
KJ672302.1	4	0.005	0.000	0.000		0.000	0.000	0.000	0.000	0.000	0.000	0.000
KJ660959.1	5	0.005	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.000	0.000
KJ572281.1	6	0.005	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.000
KC851831.1	7	0.005	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.000
KJ417904.1	8	0.005	0.000	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.000
KJ210650.1	9	0.005	0.000	0.000	0.000	0.000	0.000	0.000	0.000		0.000	0.000
KJ210642.1	10	0.005	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		0.000
KJ210640.1	11	0.005	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	

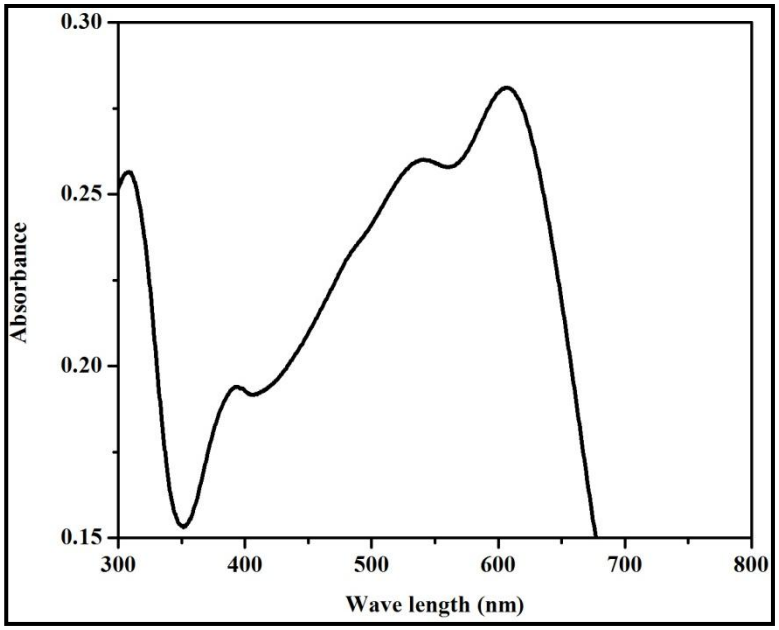

Figure 1: UV-Vis spectroscopic scan of SDM

Dessemination

International Journal

1. **Adya Das**, Susmita Mishra, Vishal Kr. Verma. "Enhanced biodecolorization of textile dye remazol navy blue using an isolated bacterial strain Bacillus pumilus HKG212 under improved culture conditions" J Biochem Tech, (2015) 6(3): 962-969
2. **Adya Das** and Susmita Mishra. "Decolorization of different textile azo dyes using an isolated bacterium Enterococcus durans GM13" International Journal of Current Microbiology and Applied Sciences ISSN: 2319-7706 Volume 5 Number 7 (2016) pp. 675-686
3. **Adya Das**, Susmita Mishra. "Removal of Textile dye Reactive Green-19 using bacterial Consortium: Process optimization using response surface methodology and kinetics study" Journal of Environmental Chemical Engineering. (Accepted)

Conferences

1. Susmita Mishra and **Adya Das**. "Decolorization of synthetic dyes using marine microorganism" International Conference on Frontiers in Chemical Engineering, Department of Chemical Engineering, NIT Rourkela, December 09-11, 2013.
2. Susmita Mishra and **Adya Das**. "Decolorization and Degradation of Reactive Green Dye using Biological Treatment" CHEMCON-2013, Institute of Chemical Technology, Mumbai, December 27-30, 2013.
3. **Adya Das**, Susmita Mishra. "Decolorization of Remazol Navy Blue and simultaneous electricity production using a developed consortium in dual chambered microbial fuel cell" International Conference on Recent Trends in Engineering and Material Sciences, Jaipur National University, March 17-19, 2016.

Article under preparation

1. **Adya Das**, Susmita Mishra. "Optimization of culture condition of a novel bacterial consortium using response surface methodology for enhanced decolorization of mixture of azo dyes and kinetic study".
2. **Adya Das**, Susmita Mishra. "Complete mineralization of azo dye Remazol navy blue in MFC-aerobic integrated system and simultaneous energy production".

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